Faculty of Biological and Environmental Sciences
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Using a combination of bioassays, bioaccumulation kinetics and mixture toxicity tests to assess the ecotoxicological risk of CCA contaminated soils in Finland

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ACADEMIC DISSERTATION IN ECOTOXICOLOGY

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Dedicated to my « **favourite uncle** » **Kosti** and **my children, Julia, Veera and Nestori**, who are the most important to me in my life and the loving memory of my **god mother Pirjo**

ABSTRACT

In this thesis we studied the effects of Chromated Copper Arsenate (CCA) compounds in Finnish forest ecosystems. The research site was located in Hartola, southern Finland. Between 1958 and 1966, there was a small wood impregnation plant which used the so-called Lahontuho K-33 impregnating agent. The aim of this thesis is to use a combination of applied bioassays, uptake and elimination kinetics and binary mixture toxicity experiments to gain better understanding of the bioavailability of Cu, Cr and As and their potential eco(toxico)logical risk at the Hartola site. This approach fits the TRIAD method, which uses a combination of chemical analyses, bioassays and field observations to assess the actual risk of contaminated soils.

The thesis contains three articles that approached the risk assessment of CCA compounds using different methods. In **article I**, bioassays were used to assess whether Cr, Cu and As are bioavailable to plants (*Lemna minor* and *Lactuca sativa*) and invertebrates (*Enchytraeus albidus* and *Lumbricus rubellus*) and whether they pose a risk to the ecosystem. **Article II** focused on the bioavailability of chromium, copper and arsenic in Hartola soils using an uptake and elimination kinetics approach with the earthworm *Eisenia andrei*. **Article III** determined the toxicity of the CCA metals, single and in binary mixtures, to earthworms (*Eisenia andrei*), in particular using reproduction as the endpoint.

Based on the studies in **article I**, it was concluded that the CCA metals in the Hartola soils cause damage to organisms and pose a risk to the ecosystem. Metals did bioaccumulate, as shown by the metal concentrations in earthworms and plants. The toxic effects obtained in bioassays correlated best with the concentration of arsenic in the Hartola soils.

In **article II**, earthworms were shown to take up and excrete chromium and copper in the same way: fast uptake and fast elimination occurred reaching steady state within 1 to 3 days. Arsenic uptake in the earthworms, however, was slow and did not reach equilibrium. Uptake and elimination rate constants were used to calculate the bioaccumulation factor (BAF), which was highest for As, supporting the conclusion of article I.

In **article III**, at low concentrations copper and chromium had a hormetic effect on earthworm reproduction, which was not seen with As. Arsenic was already toxic to the earthworms at low concentrations. Arsenic-containing mixtures also were highly toxic. The binary mixtures of CCA metals generally acted antagonistic on earthworm reproduction when tested against to the Concentration Addition model. It seems that the CCA metals react with each other, leading to a reduced toxicity in the mixtures.

The results of this thesis showed that at the study site the metals were bioavailable as shown from their bioaccumulation in invertebrates and plants, and that the risk of CCA pollution is mainly due to As. Based on our results we suggest that the TRIAD method is a useful approach to determining the potential eco(toxico)logical risk of CCA metals.

TIIVISTELMÄ

Tässä väitöskirjassa tutkittiin kromia (Cr), kuparia (Cu) ja arseenia (As) sisältävän yhdisteen (CCA-yhdiste) vaikutuksia suomalaiseen metsäekosysteemiin Hartolassa, Etelä-Suomessa. Vuosien 1958 – 1966 välisenä aikana alueella kyllästettiin sähköpylväitä Lahontuho K-33 kyllästysaineella. Tutkimusmenetelminä käytettiin valikoituja biotestejä, metallien akkumulaatio- ja eliminaatiokinetiikkaa ja seostoksisuuskokeita, joiden avulla saatiin lisää tietoa kromin, kuparin ja arseenin biosaatavuudesta ja näiden metallien mahdollisesta eko(toksiko)logisesta riskistä ekosysteemille TRIAD-menetelmää hyödyntäen. Tämä lähestymistapa sopii TRIAD-menetelmän hyödyntämiseen, koska siinä hyödynnetään kemiallisten analyysien kombinaatioita, biotestejä ja kenttähavaintoja määritettäessä pilaantuneiden maiden todellista riskiä.

Tutkimus jaettiin kolmeen osaan, joissa käsiteltiin CCA-yhdisteiden riskinarviointia em. menetelmiä apuna käyttäen. **Artikkelissa I** selvitettiin biotestien avulla, ovatko Cr, Cu ja As kasveille (*Lemna minor* ja *Lactusa sativa*) ja selkärangattomille (*Enchytraeus albidus* ja *Lumricus rubellus*) biosaatavassa muodossa ja aiheuttavatko ne riskiä ekosysteemille.

Artikkelissa II tutkittiin kromin, kuparin ja arseenin biosaatavuutta, akkumulaatio- ja eliminaatiokinetiikan avulla. Tavoitteena oli selvittää, kuinka nopeasti lierot (*Eisenia andrei*) ottavat elimistöönsä ko. metalleja ja kuinka nopeasti ne pystyvät erittämään metallin pois sen jälkeen, kun lierot on siirretty puhtaaseen OECD standardin mukaisesti valmistettuun keinotekoiseen maahan. Näiden kokeiden avulla haluttiin tarkastella tarkemmin ko. metallien biosaatavuutta ja pohtia, miten maaperän ominaisuudet vaikuttavat metallien biosaatavuuteen sekä saada lisätietoa alueen riskinarvioinnin tueksi.

Artikkelissa III selvitettiin yksittäisen metallin ja kahdesta metallista muodostettujen seosten (Cu-As, Cu-Cr, Cr-As) vaikutuksia lieroihin (*Eisenia andrei*), etenkin niiden lisääntymiseen käyttäen excel-pohjaista MIXTOX-mallia.

Artikkelin I tutkimukset osoittavat, että CCA-yhdisteen metallit aiheuttavat eliöstölle haittaa ja aiheuttavat riskiä ekosysteemille. Cr, Cu ja As kertyivät selkarangattomiin. Arseenin pitoisuus maaperässä korreloi parhaiten biotesteissä saatuihin haitallisiin vaikutuksiin. **Artikkelissa II** lierot akkumuloivat ja eliminoivat kromia ja kuparia nopeasti 1 – 3 päivässä, kun taas arseenilla lierot eivät saavuttaneet tasapainoa kokeen aikana. Tulosten perusteella voidaan todeta, että As kertyy hitaasti lieroihin. Akkumulaatio- ja eliminaatiovaiheen kineettisten vakioiden avulla lasketut biokertyvyysvakiot (BAF) vahvistivat em. tulokset. **Artikkelissa III** tuli esille, että pienissä pitoisuuksissa kuparilla ja kromilla esiintyi hormesis-vaikutus, jota arseenilla ei esiintynyt. Tämä johtui siitä, että jo alhaisissa konsentraatioissa arseeni aiheutti haitallisia vaikutuksia lieroihin esiintyessään yksin ja metalliseoksissa. Kaikki seokset osoittivat antagonisia yhteisvaikutuksia lierojen lisääntymistesteissä. CCA-metallit reagoivat keskenään, mutta binäärisissä seoksissa niiden toksisuus vähenee.

Tuloksiemme perusteella näyttää siltä, että riskinarvioinnissa käytettävä TRIAD-menetelmä, on hyödyllinen määritettäessä CCA-metallien ympäristövaikutusten todentamisessa.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Anne-Mari Karjalainen, Johanna Kilpi-Koski, Ari O Väisänen, Sari Penttinen, Cornelius AM van Gestel and Olli-Pekka Penttinen, 2009, Ecological risks of an old wood impregnation mill: Application of the TRIAD approach, Integrated Environmental Assessment and Management 5, 379 – 389.

II Johanna Kilpi-Koski, Olli-Pekka Penttinen, Ari O. Väisänen and Cornelis A.M. van Gestel, 2019, An uptake and elimination kinetics approach to assess the bioavailability of chromium, copper and arsenic to earthworms (*Eisenia andrei*) in contaminated field soils, Environmental Science and Pollution Research 26, 15095 – 15104.

III Kilpi-Koski, J., Penttinen, O-P, Väisänen, A.O. and van Gestel, C.A.M., 2020, Toxicity of binary mixtures of Cu, Cr and As to the earthworm *Eisenia andrei*, Ecotoxicology, online, 10.1007/s10646-020-02240-1.

The publications are referred to in the text by their roman numerals.
Articles I, II and III are reprinted with the kind permission of Integrated Environmental Assessment and Management by SETAC, Environmental Science and Pollution Research by Springer and Ecotoxicology, by Springer, respectively.

THE AUTHOR'S CONTRIBUTION

Article I
Karjalainen, AM., Kilpi-Koski, J., Penttinen, OP. and van Gestel, CAM. designed the study. Karjalainen, AM. and Kilpi-Koski, J. performed the experiments. Väisänen, A. analyzed the samples. Penttinen, S. focused on TRIAD approach. Karjalainen, AM., Kilpi-Koski, J., Penttinen, OP. and van Gestel, CAM. analyzed the data. Karjalainen, AM. and van Gestel CAM. wrote the article with input from all authors.

Article II
Kilpi-Koski, J. and van Gestel, CAM. designed the experiments. Kilpi-Koski, J. performed the experiments with support of van Gestel, CAM. Väisänen, A. analysed the samples and Kilpi-Koski, J. and van Gestel, CAM. analysed the data. Kilpi-Koski, J. wrote the manuscript in consultation with Penttinen, OP. and strong support of van Gestel, CAM.

Article III
Kilpi-Koski, J. and van Gestel, CAM. designed the experiments. Kilpi-Koski, J. performed the experiments with support of van Gestel, CAM. Väisänen, A. analysed the samples and Kilpi-Koski, J. and van Gestel, CAM. analysed the data. Kilpi-Koski, J. wrote the manuscript in consultation with Penttinen, OP. and strong support of van Gestel, CAM.

GLOSSARY

Additivity	Toxicity of the mixture agrees with the effect expected based on the toxicities of the individual substances
Antagonism	Toxicity of the mixture is lower than expected from the toxicities of the individual substances.
Available	The chemical fraction present in soil in an easily accessible form as determined by chemical extraction with e.g. water or a diluted salt (0.01 M $CaCl_2$)
BAF	Bioaccumulation factor, defined as the ratio of concentrations in the test organism and in the soil
BCF	Bioconcentration factor, defined as the ratio of the concentrations in the test organism and in the soil solution (pore water)
Bengal red	The dye used to colour enchytraeids helping to identify them
Bioaccessible	Any chemical fraction that is really taken up into tissues of organisms
Bioassay	Biological tests, for example with plants and animals, to determine the actual risk of field-contaminated media like soils, by assessing their toxicity or the bioaccumulation of pollutants present in the organisms
Bioavailable	Any chemical fraction that is or may become available for uptake by organisms (Peijnenburg and Jager, 2003).
CA	Concentration addition
CCA	Chromated copper arsenate
DL	Dose-level dependent deviation
DR	Dose-ratio dependent deviation
DSS	Decision Support System
EC_{10}	10 % effective concentration, the concentration reducing the measured parameter (e.g. number of juveniles produced) by 10% compared to the untreated control
EC_{50}	50 % effective concentration, the concentration reducing the measured parameter (e.g. number of juveniles produced) by 50% compared to the untreated control
Ecotoxicology	The field of research studying the harmful impacts of potentially toxic chemicals on ecosystems by combining three different diciplines : toxicology, ecology and chemistry.
ERA	Ecological Risk Assessment
EU	European Union
Heavy metal	Metal having a density > 5 g/cm^3
Hormesis model	A mathematical dose-response model that takes account of stimulated (hormetic) responses at low test concentrations
IA	Independent action
ICP-OES	Inductively-Coupled Plasma – Optical Emission Spectrometry
ISO	International Organization for Standardization

k_1	Uptake rate constant
k_2	Elimination rate constant
K_f	Freundlich sorption constant
LC_{50}	The concentration killing 50 % of the exposed test organisms
LoE	Line of evidence
LOEC	The Lowest Observed Effect Concentration, the lowest concentration tested at which the measured parameter (e.g. number of juveniles produced) differs significantly from the control
Logistic model	A mathematical dose-response model based on logistic function
MIXTOX model	Excel based model by Jonker et al. (2005) which allows for evaluating mixture toxicity data against the reference models of CA and IA
NOEC	The No Observed Effect Concentration, the highest concentration tested at which the measured parameter (e.g. number of juveniles produced) does not differ significantly from the control
OECD	Organization for Economic Co-operation and Development
Pesticide	A chemical substance that controls pest organisms.
Speciation of metals	Chemical forms (species) in which metals may occur in the environment.
SPSS	Statistical Package for the Social Sciences
Synergism	Toxicity of the mixture is higher than expected from the toxicities of the individual substances
TRIAD	A practical tool for the risk assessment of contaminated soil and sediment, making use of three lines of evidence: chemical analysis, toxicity testing and ecological observations
TU	Toxic unit
WHC	Water holding capacity
XRF	X-ray fluorescence

1 INTRODUCTION

In 1962, Rachel Carson wrote the book “Silence Spring” highlighting the fact that organic pesticides accumulate in food chains and cause great environmental effects. Eventually, this book was the start of the environmental movement, triggering people to care about protecting their environment. Step by step measures and methods of environmental protection started developing. And researchers began to use the term ecotoxicology which combines three different disciplines: environmental chemistry, toxicology, and ecology. Ecotoxicology is studying the harmful impacts of potentially toxic chemicals on ecosystems including microbes, plants and animals. The term Ecotoxicology was coined by René Truhaut in 1969 (Truhaut, 1977).

Ecotoxicology has a direct relevance for the ecological risk assessment of chemicals and contaminated sites. In ecotoxicological studies, researchers need to account for the metabolic transformation of chemicals, their uptake and elimination routes in organisms, concentration-response relationships, effects of chemicals single and in mixtures, statistical aspects etc. in order to be able to predict the effects of pollutants on higher levels of biological organisation and to define toxicity thresholds. Experimental designs need to consider relevant endpoints, test species and uncertainty of evaluation (Rudén et al., 2016).

Soil is under many pressures, like human population growth, efficient land use, pollution and climate change. Soils are contaminated by different toxicants like pesticides, oil, and metals. Soils are named contaminated when the concentrations of pollutants, contaminants or chemicals are high enough to pose a risk to ecosystems. Contaminants, like metals, may contaminate air, water and soil ecosystems through natural and/or anthropogenic routes. In Figure 1 soil pollution sources are illustrated.

Natural sources of metals are erosion of bedrock rich in metals or volcanic activity (mainly relevant for Hg). Metals exist in ores in different chemical forms. Weathering releases metals from the bedrock into the environment (Khan et al., 2011). In Finland around 47 metallogenic areas can be found with active or closed mines. Of these, 10 areas contain ferrous metals, 11 areas precious metals, 8 areas have copper, zinc and/or lead, 4 areas have potential for mining metals used in advanced technologies (mainly micro-electronics) and 3 areas contain uranium (Eilu, 2012).

Anthropogenic metal pollution sources are often point sources like mining, traffic, smelters and emission of combustion by-products (Figure 1). Contamination of air, water and soil varies from place to place (Khan et al., 2011). Finland has a long history of mining, since 1540 when iron ore mining was started. Copper, nickel, cobalt, zinc and ores of lead chromium, vanadium and iron have been mined and processed for the Finnish metal industry (Geological Survey of Finland, 2019).

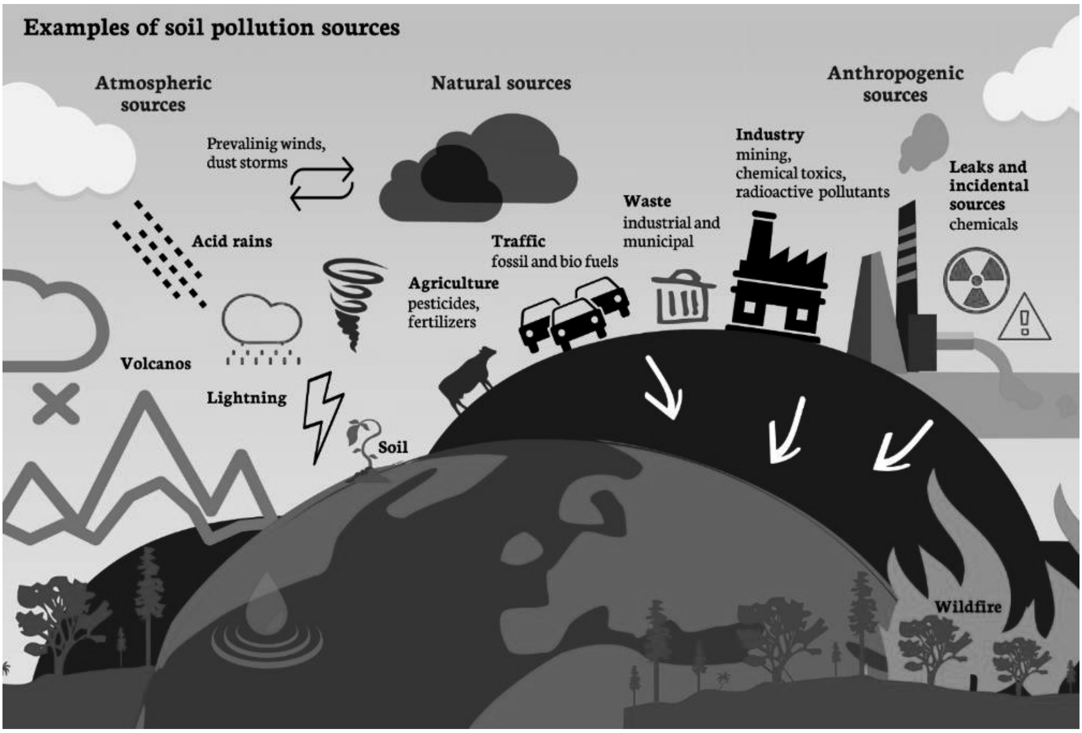

Figure 1 Soil pollution sources. Figure prepared by Anri Vuori© (2019).

Among other anthropogenic sources, mining activities can cause metal contamination in areas nearby or far away because of transport through the air or leakage into waterways. For example, in eastern Finland, near Talvivaara mining, there was the most serious environmental conflict in Finland for many years. Step by step the environmental crisis deepened and at the end of 2012 there were serious metal leakages (nickel (Ni)) from a gypsy sediment basin to the surrounding environment, also to rivers and lakes (Sairinen et al., 2017) causing a ban of water use. Metal emissions into water systems will be diluted but it can be spread over a wide area causing accumulation into the food chain.

Total metal concentrations in soils, however, do not necessarily show the potential risk for the ecosystem. Metals need to be present in the soil solution compartment to be bioavailable to soil organisms. Soil properties play an important role influencing the bioavailability of metals and in that way their risk to organisms and ecosystems.

1.1 Metal pollution in Europe

In Europe, metal contamination is mainly due to industry, mining, metal processing and the energy production sector. In Europe, in 2016 an estimated 978 facilities were responsible for the emission of metals to air, but only 18 of these facilities produced more than half of the emissions. These estimates were made by the European Commission applying the USEtox model. Since 2010 emissions of metals have been reduced by 39 %. In 2016, the USEtox model indicated that 59 % of the environmental pressure came from metal production and processing and 20 % from thermal power stations (European Environment Agency (EEA), 2019).

Based on inventory of 39 European countries it has been estimated that there are about 2.5 million potentially contaminated sites (PCS) in Europe where the soil contamination is suspected but not verified. About 14 % (340 000) of 2.5 million (PCS) sites are verified where the soil contamination is confirmed. According to the European Commission Joint Research Center, an average 42 % of the total expenditure for the management of Contaminated sites comes from public budgets ranging from 25 % (Belgium, Flanders) to 90 % (Estonia). Cost estimate for the management of contaminated sites is around 6 billion euros annually (van Liederkerke et al., 2014).

According to data collected through a European Network, the main contaminants are mineral oil and metals contributing 60 % to soil contamination (Panagos et al., 2013). The LUCAS Topsoil Survey collected over 23 000 topsoil samples (upper 20 cm) from the European Union (EU) Member States (EU-28, except Croatia) to provide a coherent baseline topsoil database for continental scale monitoring. We took a closer look to copper, chromium and arsenic because they are used in wood preservatives known as CCA compounds. Although usage of CCA compound is now prohibited, its use has resulted in sites which are contaminated by these compounds and need remediation.

In the LUCAS Topsoil Survey, copper was found only at low concentrations in the Northern part of Europe. Higher concentrations of Cu were detected in the Mediterranean area. There were six regions with predominant geology-derived Cr accumulation, mainly in the Mediterranean area, Greece and Italy. Arsenic levels were < 20 mg/kg in > 95% of the samples. Northern European regions were basically free of As but in mountain areas, like the Alps, higher As levels were found (Tóth et al., 2016). Figure 2 shows the concentrations of Cu, Cr and As in top soils of the EU Member States (EU-28, except Croatia).

Finland has a long history of mining and processing copper. The biggest deposit of copper was found in Eastern Finland. Processing of copper has been done in Western Finland for almost 80 years now. Unfortunately, during these years there have been emissions to surrounding areas causing Cu contamination. As seen from Figure 2, copper concentrations in Finnish soils are mostly less than 10 mg/kg. Average concentration of chromium in soils is 10 – 90 mg/kg (Salminen and Lampio, 1995), the higher concentrations are found in the Northern part of Finland. Stainless steel, leather tanning, metal plating and chemical industries are using chromium in their processes (Mukherjee, 1998). Usually, in Finnish bedrock the arsenic concentration is less than 10 mg/kg, but some geological areas, like ore deposits, contain higher than average concentrations of arsenic (Eilu et al., 2012).

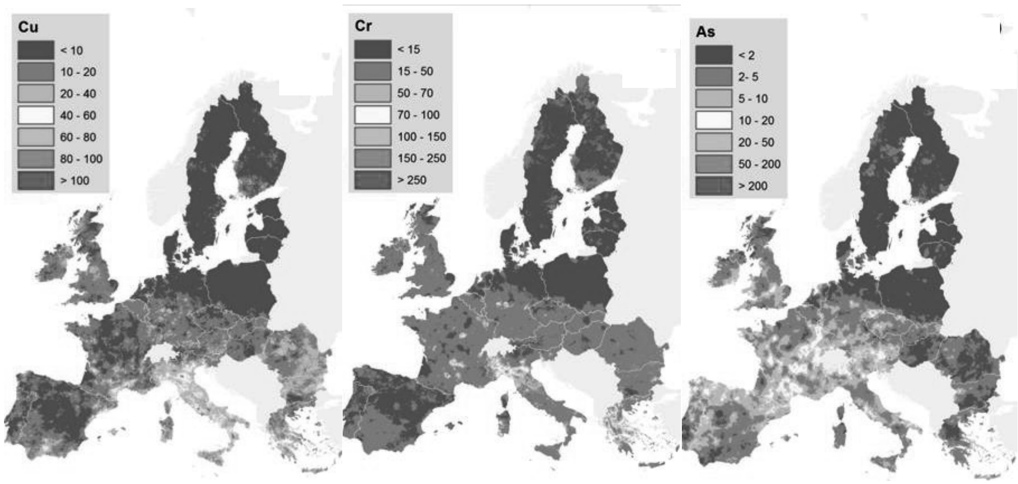

Figure 2 Concentrations of Copper (Cu), Chromium (Cr) and Arsenic (As) (mg/kg dry soil) in European top soils (Tóth et al., 2016).

In Finland the average (and ranges of) background values for Cu, Cr and As are 22 (5 – 110), 31 (6 – 170) and 1 (0.1 – 25) mg/kg, respectively (the Finnish Government Decree on the Assessment of Soil Contamination and Remediation Needs (214/2007), 2007). In Finland, the Geological Survey of Finland (GTK) provides geochemical surveys and mapping information on metal levels in soils (Jarva, 2016).

1.2 Soil pollution in Finland

In Finland, soil pollution has been studied since the 1980s. Finland has around 24000 contaminated sites, including 880 sites used for wood salt impregnation and sawmills (Pyy et al. 2013). Contaminated soils, already remediated soils or soils suspected of being polluted have been gathered in the database MATTI. About 13 000 contaminated sites are located nearby immediate living areas. Figure 3 highlights the polluting industries in Finland (Finnish Environment Institute SYKE, 2019).

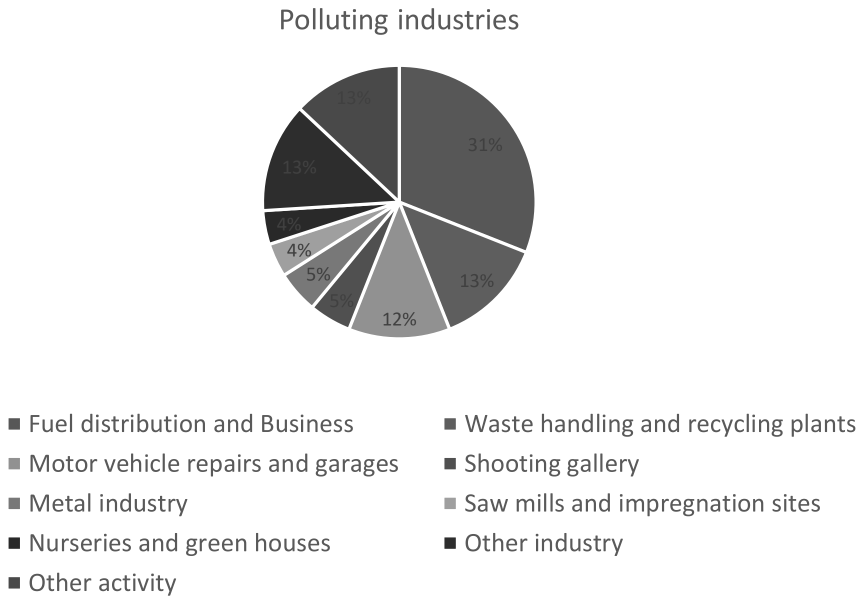

Figure 3 Polluting industries in Finland as identified by the MATTI database (Finnish Environment Institute SYKE, 2018).

Annually in Finland 50 – 100 million euros are used to investigate and remediate contaminated sites. Causes of soil contamination are inadequate waste management, illegal landfill, emissions from industry, and chemical and oil storage. Significant savings, more than 20 million euros yearly, could be achieved using more advanced, sophisticated and sustainable methods (Finnish Environment Institute SYKE, 2017). For example, in Soilia the Soil Research Center (located in Lahti, southern Finland) can be used for testing new methods for soil remediation.

Between 1986 and 2013 nearly 4900 remediation decisions were made by Finnish environmental authorities. It has been evaluated that with this speed 11 000 sites will be remediated after 100 years and it will cost 4 billion euros (Finnish Environment Institute SYKE, 2019). Often, the most common remediation activity is to excavate the contaminated soil and deliver it to a hazardous waste disposal power plant where it is burned. Annually almost 1 500 000 t of excavated contaminated soil is stored, handled or placed for final disposal. Utilization of ecotoxicological studies, like bioassays assessing actual toxicity of the contamianted soils, and mixture toxicity and toxicokinetics studies to determine bioavailibility, bioaccumulation and the role of individual pollutants in causing toxicity, could be helpful in considering alternative remediation methods for contaminated soils.

1.3 Bioavailability of metals

Despite all anthropogenic sources of metal pollution, metals also occur in soils naturally. Metals can be essential or non-essential for animals or plants. In Finland, metal concentrations in the soil are compared with background levels when defining threshold and lower and upper guideline values, as done by the Ministry of the Environment, Finland (2007), when evaluating the risk of metal contamination. Upper guideline values for As, Cr, and Cu concentrations are 100, 300, and 200 mg/kg dry soil, respectively *(*Ministry of the Environment, Finland, 2007). Metal concentrations above these defined guideline values may cause a risk to human or environmental health, e.g. by the accumulation in food chains. Not all metal has to contribute to the risk as only a fraction of the total concentration may be available for uptake and causing effects. So, risk limits based on total concentration are not sufficiently informative on actual risks.

Bioavailability and the consequent toxicity of metals depends on the metal itself, the exposed biological species and its ability to regulate metal uptake and excretion, and the environmental compartment where the organism lives (Peijnenburg and Jager, 2003). Additionally, the organism size, receptor(s), specific pathophysiological characteristics, the metal route of entry, the duration and frequency of exposure, the dose and the exposure matrix may also impact bioavailability (Allen et al., 2002). According to Peijnenburg et al. (1997), bioavailability should be considered as a dynamic process containing two distinct phases: a physico-chemically driven desorption process and a physiologically driven uptake process. The first one relates to the release of metals from the **soil solid phase into the soil solution**, the seoncd one to the uptake of metals from the soil solution by soil organisms and their distribution throughout the body. All these processes are dynamic. Soil properties like **pH** (van Gestel and Hensbergen, 1997), redox potential (Masscheleyn et al., 1991)**, Ca concentration and organic and clay matter content** *(*Lin and Puls, 2000*)* may affect the bioavailability of metals and their kinetics of uptake and elimination in organisms and the development of body concentrations with time (Vijver et al., 2003). Figure 4 shows the key components of a dynamic approach of metal bioavailability.

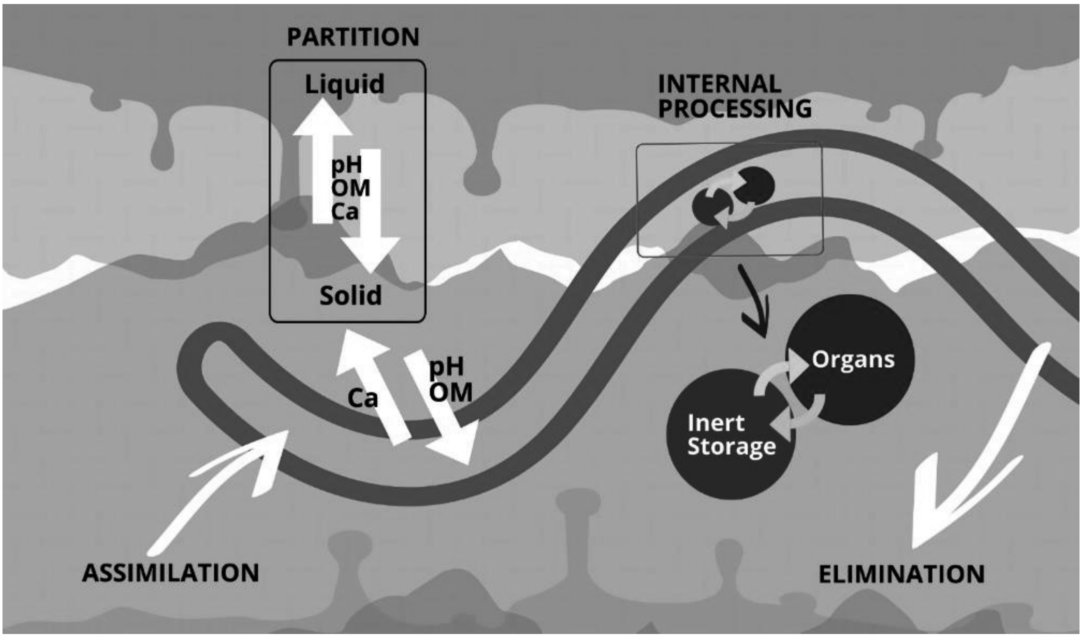

Figure 4 Biodynamic approach of metal bioavailability (modified from Lanno et al., 2004, illustrated by Anri Vuori 2019©).

1.4 Bioassays

Bioassays may provide a direct measure of the possible ecotoxicological risk of contaminated soil, and give information on the bioavailability of metals. Bioassays show the direct risk of chemical pollutants to soil organisms. From a biological point of view, bioassays therefore may be more suitable tools for assessing the actual bioavailability of metals in contaminated soils compared to chemical measurements of total or available metal concentrations. The results of the bioassays used in this study, like seed germination, root elongation and plant growth inhibition, may be directly affected by the bioavailable metal levels in the soil rather than by total metal concentrations (Sizmur and Hodson, 2009).

1.5 Uptake and elimination kinetics

Since the toxicity of metals depends on the concentration in the body, uptake and elimination kinetics are important and relevant tools for evaluating the bioavailability of metals. Moriarty and Walker (1987) concluded that uptake, metabolism and elimination studies are needed to predict the bioaccumulation of chemicals in ecosystems. They pointed out that such studies are complex and require a great deal of data to enable estimating uptake and elimination rate constants. Nevertheless, gradually researchers paid increasing attention to uptake and elimination kinetics studies. Van Straalen et al. (2005) suggested that the rate at which a metal (like zinc in their study) is entering the organism might be more important than the concentration inside the organism. They emphasized the importance of uptake rate as an indicator of bioavailability (van Straalen et al., 2005). Since the toxicity of metals depends on the concentration in the body of organisms, it is important to know their uptake and elimination kinetics. Uptake kinetics show us if exposure time is adequate; only when steady state is reached, this is the case.

1.6 Mixture toxicity

Historically contaminated sites may contain many contaminants forming complex mixtures. This may also be the case for CCA-contaminated soils, where the salts of the different components may have interacted and reacted to form complexes, resulting in different ratios of the CCA components compared to freshly exposed soils. In mixtures, metal concentration ratios in the body are crucial for determining and understanding toxicity. When the uptake and elimination kinetics of metals differ, this will lead to differences in metal ratios in the body of the exposed organisms, like for instance in the case of CCA contamination. This also may be affected by differences in the bioavailability of the metals in the mixture (compared to the single metal exposures). Interaction between metals can cause greater or smaller toxic effects than expected from the toxicity of the single metals. If the mixture is more toxic than expected, the interaction is called synergistic; if it is less toxic the interaction is called antagonistic (van Gestel et al., 2011).

1.7 Soil organisms

Soil organisms are important and relevant species to use in ecotoxicological experiments, like in uptake and elimination kinetics studies, and in tests studying mixture toxicity using effects on survival, growth and reproduction. Soluble metals in the soil solution can be adsorbed onto soil particles or taken up by soil organisms, like by earthworms via dermal or gut routes. Earthworms are at the lower level of food chains, which gives the possibility to evaluate the risk for the whole food chain and part of the ecosystem. Metals may be stored or excreted by soil organisms (Lanno et al., 2004).

Soil invertebrates are used for toxicity tests because they fulfil several criteria: they represent the ecosystem at several levels, their responses are measurable, and tests can be run in the same test media. The Organization for Economic Co-operation and Development (OECD) has started to develop guidelines for toxicity test in the 1960s and it took a couple of decades to have the first toxicity test (OECD 1984) on short-term responses like the survival of earthworms. Toxicity tests have been developed since the 1980s to include reproduction and growth of earthworms, enchytraeids, springtails and several other groups of soil invertebrates (van Gestel, 2012).

In this study, we used the earthworm *Eisenia andrei* and the potworm *Enchytraeus albidus* because there are standardized toxicity tests and laboratory cultures available for these species. In addition, *Lumbricus rubellus* was collected from a non-polluted and forested area in Lahti Finland; this species is acclimated to the Finnish forest environment.

1.8 Risk assessment of soils

The potential risk of metals in soil for man and the environment depends on their bioavailability (van Gestel, 2008). The quantity and quality of the soil organic carbon play an important role in controlling the bioavailability and hence the risk of metals in the soil (Jensen and Mesman, 2006). The history of contamination and ageing may lead to a reduction of the bioavailability of metals, but this phenomenon is not sufficiently covered in the risk assessment. As a consequence, actual risk is often over-estimated and improved tools for measuring the bioavailability fraction of contaminants are needed. Peijnenburg et al. (1997) stated that dynamic biological measures of bioavailability can be added to the risk assessment of soil contamination. Van Straalen et al. (2005) confirmed this and suggested that indicators of bioavailability can be derived from the rate at which organisms take up contaminants from the environment. By reducing the bioavailable fraction with stabilization techniques, like the addition of soil amendments, the risk to the environment can be limited (Gonzales et al, 2013). In addition to bioavailability, also heterogeneity and spatial variability of the contamination as well as the ecology of soil organisms should be taken into account in the risk assessment of soil contamination, as was shown for earthworms by Marinussen et al. (1997).

Contamination of soils is a challenge in almost every country and asking for appropriate remediation technologies and Ecological Risk Assessment (ERA) procedures. ERA is often very complicated with many variables of concern and can be time consuming and expensive (Peijnenburg et al., 2007). Hingston et al. (2002) pointed out that a careful design of standardized protocols is necessary if accurate and realistic risk assessments are to be made. Risk assessment also requires easy, time and money saving methods and models (Saxe et al., 2011). The TRIAD approach can offer a helpful protocol to evaluate the risk of contaminated sites. The TRIAD approach is considered a cost-efficient method, which combines three lines of evidence: chemistry, toxicology and ecology. These three lines of evidence form different levels of information which can be structured in a Decision Support System (DSS) that can be used in every tier of the risk assessment (Jensen and Mesman, 2006). When evaluating the ecological risk of metals, it is recommended to combine bioassays with chemical analyses because chemical analyses alone don't show the behavior of the metals in the environment (Wang et al., 2018).

1.9 Background of the thesis

The main driver for performing this study on an old wood impregnation site was that there is little information about the bioavailability of CCA metals in mixtures, and therefore great uncertainty exists about their potential risk in soils. Out of the 880 sites used for wood salt impregnation and saw mills in Finland (Pyy et. al., 2013), about 200 sites were identified as polluted by the inorganic wood impregnation chemical chromated copper arsenate (CCA) (Haavisto, 2003). On 71 sites, remediation activities have taken place (email from Matti Silvola on 17th of April 2020).

In this thesis, we used a combination of applied bioassays and fundamental research (uptake and elimination kinetics, binary mixture experiments) that together aims at shedding more light on the risk of CCA contaminated soils.

1.9.1 Study site in Hartola, southern Finland

In this thesis we have studied an old wood impregnation site in Hartola, southern Finland which is contaminated by chromated copper arsenate (CCA) mixtures. The forest at the site (Hartola, Southern Finland (68⁰17´820N/34⁰44´030E)) was a 60-yr-old Norway spruce (*Picea abies* L.) stand of *Oxalis-Myrtillus* site type (Cajander, 1949). Horsetails (*Equisetum spp.*) were growing quite abundantly on the site.

At the site, wood logs were preserved with K-33, which contained 34.0% As(V)Oxide, 26.6% Cr_2O_3, 14.8% CuO and 24.6% water. During the period 1958 – 1966, approximately 2500 wood logs were preserved per year, using 8400 liters K-33 liquid annually. After treatment, the wood logs were dried in the area for three days. When finishing impregnation actions in autumn, leftover wood impregnation liquid was discarded by pouring onto the soil, leading to contamination of the soil and ground water.

1.9.2 CCA wood preservatives

Chromated Copper Arsenate (CCA) use in wood preservatives began in 1950 with the application of Lahontuho K33 (Viitasaari, 1991). In Sweden, it was known as Boliden K33, which became the most widely used CCA formulation. K33 was marketed by many companies around the world under various trade names (Richardson, 1993). The CCA compounds are divided into A, B and C type compounds according to the amount of arsenic, but they also differ in solubility (Viitasaari, 1991). Until the end of 1982, the CCA wood preservatives used in Finland were of the type B compound. After that, type C compounds were introduced. Until 2003, CCA wood preservatives were the most popular compounds used for wood impregnation worldwide because they were effective because of the toxicity of copper and arsenic to fungi and insects (Lebow, 1996).

However, the CCA metals have been shown to accumulate in the environment under or near CCA treated wood (Stilwell and Gorny 1997). Leaching of CCA preservatives into the environment depends on weather conditions and soil characteristics (Balasoiu et al., 2001; Stilwell and Gorny 1997). Reduction of Cr(VI) to Cr(III) is the main driver of a series of reactions in the fixation of CCA complexes, resulting in the insolubilization of CCA. Adsorption and fixation reactions reduce the leachability of Cu, Cr and As into the environment but environmental factors like pH and temperature may also affect their leachability (Hingston et al., 2001). The leached CCA metals are expected to adsorb quickly to soil particles, but may be desorbed into the soil solution after rainfall or irrigation events (Leduc et al., 2008). CCA leaching generally increases with the age of CCA-handled timber (Katz and Salem, 2005). As a consequence, many terrestrial and aquatic ecosystems are contaminated with leachates of CCA treated wood.

1.9.3 Bioavailability of chromium, copper and arsenic

The bioavailability of Cu, Cr and As and their partitioning in the field-contaminated soils are influenced by soil properties like soil pH, organic matter and clay content. All three metals act differently in soils and invertebrates. Copper is an essential metal for organisms and can have beneficial effects at lower concentrations (García-Gómez et al., 2014), among other because it plays an important role in numerous enzymes in all living organisms (Fisker et al., 2013).

Chromium is essential in some metabolic process. In soil, Cr has two stable oxidation states: +III and +VI. Cr(VI) is more toxic and mobile than Cr(III). The reduction of Cr(VI) to Cr(III) happens at low pH when a suitable reducing agent is present (Wittbrodt, 1995), while the oxidation of Cr(III) to Cr (VI) is catalysed by manganese oxide (MnO_2) (Reijonen and Hartikainen, 2016). Cr(VI) was more toxic for *E. fetida* than Cr(III): LC_{50} for Cr(III) was seven times higher than for Cr(VI) (Sivakumar and Subbhuraam, 2005).

As is a metalloid with characteristics of metals and non-metals. In soil, inorganic arsenate As(V) and arsenite As(III) are the most common speciation forms of As. According to Masscheleyn et al. (1991), As (V) is reduced to the more mobile and toxic As(III) because it is an effective electron acceptor in the microbial mineralization of organic matter. They also noticed that the solubility of As is controlled by ferrum Fe(III). In turn manganese(IV) oxides affected the oxidation of As(III) to As (V). Redox and pH also influence the speciation and solubility of As. At lower pH and high redox potential As is mainly in the As(V) form, while at high pH and low redox potential As(III) is the dominant form (Masscheleyn, 1991). The bioavailability of arsenic is difficult to measure because it can be present in cationic or anionic forms. In earthworms more As (III) than As (V) was found because As (V) is reduced to As (III) (Lee and Kim, 2013).

1.9.4 Enchytraeids and earthworms

Enchytraeids and earthworms are widely used as test species in ecotoxicological experiments. Enchytraeids are small, soil-dwelling annelids (Oligochaeta, Annelida). They are one of the key species in terrestrial ecosystems contributing to decomposition and soil bioturbation (Didden, 1993). They live in very close association with the soil pore water and are exposed via dermal, intestinal and respiratory routes (Römbke, 2003). Enchytraeid are easy to culture and maintained in laboratory conditions. There are standards tests for enchytraeid toxicity testing: ISO 2004 (ISO 2004) and OECD 220 (OECD, 2004). The test duration for the *E. albidus* reproduction toxicity test is 6 weeks and its generation time is around 33 days (OECD, 2004). Enchytraeids have been used in different experimental designs on freshly spiked and field-contaminated soils, like in avoidance (Amorim et al., 2008) and reproduction tests (Castro-Ferreira et al., 2012), and testing single chemicals and mixtures (Lock and Janssen, 2002a).

Earthworms are the key players in the soil, having a very important role as ecosystem engineers (Römbke et al., 2005). Earthworms have mostly beneficial impacts on soil structure, soil chemistry and organic matter decomposition, but they can also have opposite effects, like erosion due to removal of surface litter (Römbke et al., 2005). Earthworms can be divided into three ecological categories: epigeic, anecic and endogeic. Epigeic species, like *L. rubellus* and *Dendrobaena octaedra*, live on top of the mineral soil layer where they inhabit the organic litter layer. They generally are reddish and have a short life cycle. Also the commonly used ecotoxicological test species *Eisenia fetida* and *E. andrei*, although more abundant in dung and compost heaps rather than in soil, are considered epigeic species. Anecics (for example *Lumbricus terrestris*) live in permanent vertical burrows in the mineral soil layer. They are large, dark on the dorsal site and slow movers coming to the soil surface to feed. They have a very long life-cycle. Endogeics like *Aporrectodea caliginosa* and *Allolobophora chlorotica* live in the mineral soil layers making non-permanent burrows, where they mainly feed on root exudates. They move slowly and have intermediate life cycles (Römbke et al., 2005).

Earthworms are used in different types of experiments and test designs, for example to assess the bioavailability of metals (Peijnenburg et al., 1997; Peijnenburg et al., 1999; Sizmur and Hodson, 2009; Spurgeon et al., 2011) or organic compounds (Mangala et al., 2009). Earthworms are also used in toxicokinetic studies (Van Gestel et al., 1993; Peijnenburg et al., 1999; Kilpi-Koski et al., 2019), and in mixture toxicity tests (Gomez-Eyles et al., 2009; van der Geest et al., 2000; van Gestel et al., 2011; Natal-da-Luz et al., 2011). And they are used to evaluate the ecological risk of contaminated soils in different ecosystems (Karjalainen et al. 2009; Lock and Janssen, 2001).

Metals enter the earthworm by intestinal or dermal uptake from soil and/or pore water. Copper and lead use mainly the dermal route while cadmium and zinc are taken up by ingestion. When metals enter via drinking or soil and food ingestion, gut conditions influence metal speciation and metal uptake. The dermal route mainly concerns metals taken up directly from pore water (Vijver et al., 2003). Sizmur and Hodson (2009) proposed a conceptual model of how earthworms can impact metal chemistry in soils and how this relates to different routes of metal uptake (Figure 5).

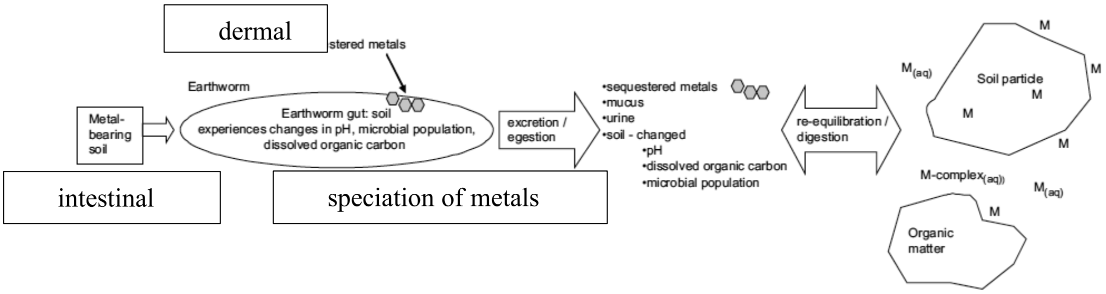

Figure 5 Potential mechanisms of how earthworms can impact soil metal chemistry and how this relates to routes of metal uptake. Modified from Sizmur and Hodson (2009) by Johanna Kilpi-Koski.

After uptake, earthworms can sequester, store and/or excrete metals depending on their properties. Some metals are essential (Veltman et al., 2007; Devillers, 2009) and earthworms can store or even regulate their internal concentration to a fairly constant level. Some metals accumulate in earthworms and can cause effects on survival (Spurgeon et al., 1994; Khalil et al., 1996), growth (van Gestel et al., 1991; Spurgeon and Hopkin, 1996), sexual development (van Gestel et al., 1991; Spurgeon and Hopkin, 1996), and reproduction (Spurgeon and Hopkin, 1996; Khalil et al., 1996; Kilpi-Koski et al., 2019). Earthworms may develop resistance to some metals, upon long-term exposure, like demonstrated for Cu (Langdon et al., 2001b) and As (Langdon et al., 1999). Better understanding of the ecology of soil invertebrates is needed to enable predicting ecosystem effects and trying to understand exposure in the field (van Gestel, 2012).

2 AIMS OF THE THESIS

There are several sites in Finland that are contaminated with chromated copper arsenate (CCA) compound. Unfortunately, not much is known about the bioavailability of CCA metal or their toxicity in mixtures predicting the risk to biota and the whole ecosystem. For that reason, the aim of the present thesis was to determine the ecotoxicological risk of chromated copper arsenate (CCA) compound used in an old wood impregnation site in Hartola, southern Finland.

To reach this aim the research was carried out with the following steps:

In article I a battery of acute ecotoxicological tests was applied to the contaminated field soil to evaluate the toxicity and bioaccumulation potential of chromium, copper and arsenic in *Lactuca sativa*, *Lemna minor*, *Lumbricus rubellus* and *Enchytraeus albidus*. To assess the ecological risk, the TRIAD approach was used.
Our hypothesis was that the metals in soils contaminated by former wood impregnation activities do cause adverse effects on the test organisms.

In article II the aim was to determine the bioavailability of chromium, copper and arsenic to earthworms along a concentration gradient to provide a basis for the ecotoxicological risk assessment of CCA-contaminated field soils. Uptake and elimination rate constants of Cr, Cu and As were determined to assess their bioaccumulation in the earthworm *Eisenia andrei*.
Our hypotheses were that the uptake kinetics in *Eisenia andrei* are different for Cr, Cu and As, and do provide insight into the bioavailability of these metals in CCA-contaminated soils.

In article III the aim was to assess the toxicity to the earthworm *Eisenia andrei* of binary mixtures of Cu, Cr and As and analyse possible interactions of the metals in the mixtures by applying the MIXTOX model of Jonker et al. (2005). Mixture effects were compared to effects of the single metal, and related to total and available concentrations in the soil.
Our hypotheses were that the metals As, Cr and Cu in binary mixtures would be more toxic than single metals alone in affecting earthworm survival, growth and reproduction. This assumption of synergistic interactions between the CCA metals is based on the dissimilarity in their modes of action and the differences in toxicokinetics in *Eisenia andrei* found in the study reported in article II (Kilpi-Koski et al., 2019).

3 MATERIALS AND METHODS

More detailed descriptions are given in original articles.

3.1 Description of the study area

The study site in Hartola is approx. 100 m x 150 m. K-33 containing chromated copper arsenate (CCA) liquid diluted with water was sprayed with pressure into two impregnation tubes of 12 m x 30 cm and one of 16 m x 3.1 m. The study site was divided into 15 squares (article I) and 4 areas (article II) based on the concentration gradient identified: control (C), low (L), medium (M) and high (H). Indicative concentrations were measured with a field-portable X-ray fluorescence meter (XRF) (Karjalainen et al., 2009). Figure 6 shows the location of the study site Hartola, Finland and a schematic design of the soil sampling.

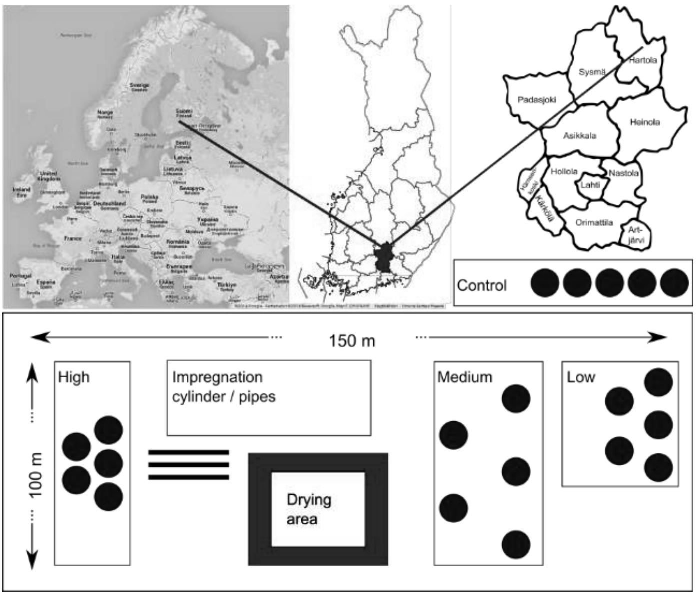

Figure 6 Location of the chromated copper arsenate (CCA)-contaminated field site near Hartola, Finland and schematic outline of the locations where soil samples were taken for the bioaccumulation tests with earthworms described in article II.

3.2 Acute ecotoxicological tests to assess toxicity and bioaccumulation of chromium, copper and arsenic (article I)

3.2.1 Sampling

In this study, we had twelve study squares (1 m^2) along a pollution gradient determined from both the humus and mineral layers with a field-portable X-ray fluorescence meter (XRF) and knowledge about the previous commercial activities. At the low end of the concentration gradient, a reference site (block 5, squares 13 – 15) was established. From each square we took three soil samples from the top 15 cm soil layer with a core sampler (Ø 50 mm) and stored them in plastic bags. Water from two wells in the study area was collected with a Limnos water sampler and stored in acid-washed plastic bottles.

At the site horsetail was growing abundantly. Roots and stems of horsetail specimen were taken with a spade and stored in paper bags to assess arsenic bioaccumulation (Wong et al., 1999). Additional soil samples were also collected from these squares for chemical and physical analyses. Detection limits for As, Cr, and Cu on the XRF meter ranged between 1 and 10 mg/kg dry soil.

3.2.2 Selected bioassays

In article I (Karjalainen et al., 2009), several bioassays were applied to assess the environmental impact of chromated copper arsenate (CCA) metals in soils from Hartola, Finland. In the bioassays, plants (*Lemna minor*, *Lactuca sativa*), earthworms (*Lumbricus rubellus*), and enchytraeids (*Enchytraeus albidus*) were exposed to contaminated soil, and/or to aqueous extracts or well water from these soils.

Phytotoxicity tests – seed germination and root elongation of Lactuca sativa

Well water and contaminated soil were used in the seed germination and root elongation test with *Lactuca sativa* that followed Schultz et al. (2004). On top of a layer of 40 g quartz sand placed in plastic petri dishes, 20 seeds were set and covered with another 20 g of quartz sand. Three replicates were prepared, which were moistened with 100% well water or its dilutions in de-ionized water or with 15 ml of de-ionized water (controls). Contaminated soil was tested in the same way, with the exception that the growth media consisted of 100% soil samples and their dilutions with crushed quartz sand, moistened with 15 ml of deionized water. The total exposure time was 7 d (2 d dark, 5 d in a light-dark cycle). After 7 d, the percent germination was determined, and the lengths of the roots were measured.

Growth inhibition test with duckweed, Lemna minor

The growth inhibition test with duckweed (*Lemna minor*) followed ISO Guideline 20079 (ISO, 2005), with slight modifications. A local strain of *L. minor* obtained from a pond in Päijät-Häme, Finland was used for testing. The test was conducted on 100% aqueous soil extracts with three replicates and on its dilutions, and also on dilutions of well water. Samples were incubated in the dark at 20 °C. Growth rate (r) per day for the leaves and roots and percent growth inhibition (%I) of *L. minor* were calculated according to ISO (2005).

Soil invertebrate tests with the earthworm Lumbricus rubellus *and the enchytraeid* Enchytraeus albidus

The earthworm *Lumbricus rubellus* was chosen for the experiment because it was used in many studies (Langdon et al., 2001 a, 2001b; Ma, 2005) and it was available. *L. rubellus* were collected from a non-contaminated forested area in Lahti, Finland and kept in soil from a non-contaminated area near Lahti, Finland. Before the onset of the experiments, the soil samples were moistened to 60% of the water holding capacity (WHC). Ten earthworms of equal size were exposed to a total of 30 samples, two samples from each square. Earthworm survival was assessed after 14 d and 28 d by hand sorting the soils and counting surviving specimen.

Dr. Eija Schultz from the Finnish Environment Institute (SYKE) kindly provided a culture of enchytraeids (*Enchytraeus albidus*) for use in toxicity tests following OECD guideline 220 (OECD, 2004), including 21-d lethality and 42-d reproduction assessment. After elimination of any soil invertebrate species (Bardgett et al., 1998) and stabilization of the field soils, the soil samples were wetted to 80% of the WHC and ten equally sized adult *E. albidus* were added. After 21 d, the surviving adults were calculated and removed. The enchytraeid reproduction test was continued, and terminated after 42 d. The juveniles produced were fixed with ethanol (99.5%), and a few drops of Bengal red were added to ease the counting of juveniles (OECD, 2004).

3.2.3 Metal analysis in oligochaetes

The enchytraeids and earthworms collected from these exposures were allowed to void their gut in petri dishes lined with moist tissue paper for 24 h. After that they were rinsed with de-ionized water, and dried for 62 h at 60 °C. The dried specimens were digested in a 1:1 ratio of de-ionized water and aqua regia (HNO_3:HCl, 1:3) and heated in a water bath (70–80 °C) until the tissue had disintegrated (T. Lukkari, personal communication 2005, Ramboll, Mikkeli, Finland). The samples were cooled and filtered over Schleicher and Schuell 0.45-µm membrane filter into acid-washed bottles. The digests were analysed for As, Cr, and Cu by Inductively-Coupled Plasma – Optical Emission Spectrometry (ICP-OES; Perkin Elmer Optima 4300DV).

3.3 An uptake and elimination kinetics approach to assess bioavailability of chromium, copper and arsenic (article II)

The metal uptake and elimination kinetics experiments were performed on soils from four test sites at Hartola, Finland: High (H), Medium (M), Low (L) and Control (C) (see Figure 6). The concentration gradient for the sampling design was improved based on results of Karjalainen et al. (2009). The soils used in the experiments were analyzed for particle size distribution by laser size grain analysis following Konert and Vandenberghe (1997). The tests lasted for 42 days, and consisted of an uptake phase in test soil from the Hartola site followed by an elimination phase in clean soil. Adult earthworms (*Eisenia andrei*) were used, which were taken from a culture at the Vrije Universiteit Amsterdam. The soils were tested at a moisture content of 50% of their water holding capacity. In the uptake phase, six replicate earthworms were sampled from each soil at days 0, 0.5, 1, 4, 8, 15 and 21. After 21 days, the remaining earthworms were taken from their respective soils, rinsed with water and transferred to non-contaminated OECD artificial soil for the elimination phase. Also during the elimination period, six replicate earthworms were sampled at days 0.5, 1, 2, 4, 8, 15 and 21. Sampled earthworms were rinsed with water to remove adhering soil particles, placed on moist filter paper to void their gut for 24 hours, weighed, frozen and freeze dried for metal analysis.

3.3.1 Total soil concentrations of chromium, copper and arsenic

Total concentrations of Cr, Cu and As were measured by weighing about 500 mg (dw) soil into 50 ml plastic bottles and adding 10 ml aqua regia ($HCl:HNO_3$, 3:1). After sonification (3 x 3 min) at a temperature of 45 – 50 °C and cooling, the samples were filtered into a 25 ml volumetric glass bottle, and diluted with high purity ELGA-water to a volume of 25 ml. Cr, Cu and As concentrations in the digests were analyzed by ICP-OES (Perkin Elmer Optima 4300DV). The procedure has been described by Väisänen et al. (2002).

3.3.2 Extractable metal concentrations in soil

About 5 g samples of the test soils were extracted with 50 ml H2O or 0.01 M CaCl2 by shaking for 2 h at 200 rpm. After settling overnight, pH was measured, and samples were 0.45 µm filtered and preserved with HNO3 for analyzing extractable metal concentrations according to Smit et al. (1997) using ICP-OES (Perkin Elmer Optima 4300DV).

3.3.3 Metal concentrations in the earthworm *Eisenia andrei*

The earthworms were digested individually in 4 ml aqua regia (HCl:HNO3 3:1). Earthworm samples were placed for 1 hour in a water bath at 70 – 80 ^{0}C. After cooling, the extract was filtered and diluted with high purity ELGA water to a volume of 25 ml (Lukkari et al., 2004). The samples were analyzed for metal concentrations by ICP-OES (Perkin Elmer Optima 4300DV) as described by Väisänen et al. (2002).

3.3.4 Calculation of uptake and elimination rate constants

In this study we used a one-compartment model (Atkins, 1969) to describe the uptake (equation 1) and elimination (equation 2) kinetics of chromium, copper and arsenic in the earthworms. The model is based on first-order kinetics which describe the change in internal concentrations over time by using two parameters (an uptake and an elimination rate constant). The uptake model (equation 1) assumed that the earthworms assimilated copper, chromium and arsenic at a constant rate for $0 \leq t \leq t_n$. The elimination model (equation 2) assumes that the earthworms excreted copper, chromium and arsenic at a constant rate once being placed in clean soil (Giska et al., 2014).

$$C_{worm} = \mathrm{C}_0 + \frac{k1}{k2} \times C_{exp1} \times (1 - e^{-k_2 t}) \quad (1)$$

$$C_{worm} = \mathrm{C}_0 + \frac{k1}{k2} \times C_{exp1} \times (1 - e^{-k_2 t}) + \frac{k_1}{k_2} \times C_{exp2} \times (e^{-k_2 (t - t_x)} - e^{-k_2 t}) \quad (2)$$

In these equations C_{worm} is the internal copper/chromium/arsenic concentration in the earthworms at time *t* (mg/kg dry body weight), C_0 the initial copper/chromium/arsenic background concentration in the earthworms at t=0 (mg/kg dry body weight), k_1 the uptake rate constant (kg soil/kg earthworm/day), k_2 the elimination rate constant (day^{-1}), C_{exp1} the copper/chromium/arsenic exposure concentration during the uptake phase (mg/kg dry soil), C_{exp2} the copper/chromium/arsenic exposure concentration during the elimination phase in clean OECD artificial soil (mg/kg dry soil), *t* the exposure time (days) and t_n the day on which the animals were transferred from the polluted soil to clean OECD artificial soil.
Both equations 1 and 2 were fitted together to obtain single values for the uptake rate constant and the elimination rate constant. IBM SPSS Statistics 21 and Microsoft EXCEL 2010 were used to fit the one-compartment model to the data for each study sites and metals. We estimated the bioaccumulation factor (BAF) for the accumulation of the metals in *E. andrei* using the uptake (k_1) and elimination (k_2) rate constants as described in equation 3 (Sharma et al., 2011):

$$BAF = \frac{k_1}{k_2} \quad (3)$$

3.4 Binary mixture toxicity of Cu-Cr, Cu-As and Cr-As (article III)

Also, in the assessment of the toxicity of binary mixtures experiments of CCA metals, the earthworm *Eisenia andrei* was used, which has been cultured at the Vrije Universiteit, Amsterdam, The Netherlands, for many years. The earthworms were exposed in modified artificial soil prepared according to OECD (1984). The artificial soil was spiked with stock solutions of K_2CrO_4 (Sigma-Aldrich ≥99.0 %), $CuCl_2*2H_2O$ (Sigma-Aldrich ≥99.0 %) and $Na_2HAsO_4*7H_2O$ (Sigma-Aldrich ≥99.0 %) in water. In this way, water content of the soil was adjusted to the right level when introducing the metals. We determined the toxicity of three binary mixtures (copper-arsenic, copper-chromium and chromium-arsenic).

3.4.1 Toxicity testing

Approx. 500 g (dw) artificial soil was weighed into 800 ml glass jars, using three replicates for each concentration and 5 controls. At the start of the experiment, 9 adult earthworms *E. andrei* were introduced into to each jar and 2 g horse dung was added for food. After 4 weeks incubation in a climate room, survival and mass of the surviving earthworms were determined. The adult earthworms were removed and the soils incubated for another 4 weeks. After this period, the number of juveniles produced was determined by placing the jars in a water bath at 60 °C. Juveniles emerging to the surface were collected and counted. Chemical analysis was performed the same way as described above, to determine total (Väisänen et al., 2002) and water and 0.01 M $CaCl_2$ extractable (Smit et al., 1997) metal concentrations in all test soils.

3.4.2 Experimental design

The experimental design for the binary mixture experiment was based on the toxic unit (TU) concept with reproduction as the endpoint, with TU defined as in equation 4:

$$TU = \frac{c}{EC50}, \qquad (4)$$

where EC_{50} is the median effective concentration causing 50% reduction of earthworm reproduction (in mg/kg dry soil), and c is the metal concentration in the mixture (in mg/kg dry soil).

Test concentrations chosen were based on the few available data on the toxicity of the three metals (Cu, Cr and As) to earthworms (Koster et al. 2006; Sivakumar and Subbhuraam 2005; Langdon et al. 2001a). As a starting point, we assumed EC_{50}s to be 200, 240 and 96 mg/kg for for effects Cu, Cr and As, respectively on the reproduction of *E. andrei*.

Nominal concentrations of the individual metals and the binary mixtures were based on expected toxic strengths of 0.25, 0.5, 1, 2 and 4 TU for Cu and 0.16, 0.4, 1, 2.5 and 6.25

for Cr and As. The mixtures tested had toxicant ratios of 1:1, 9:1, 1:9, 1:3 and 3:1. In all mixture toxicity tests, also toxicity of the single metals and the mixtures was determined simultaneously. Figure 7 shows the test design. After introduction of the metals, the artificial soil was equilibrated for three weeks before starting earthworm exposures.

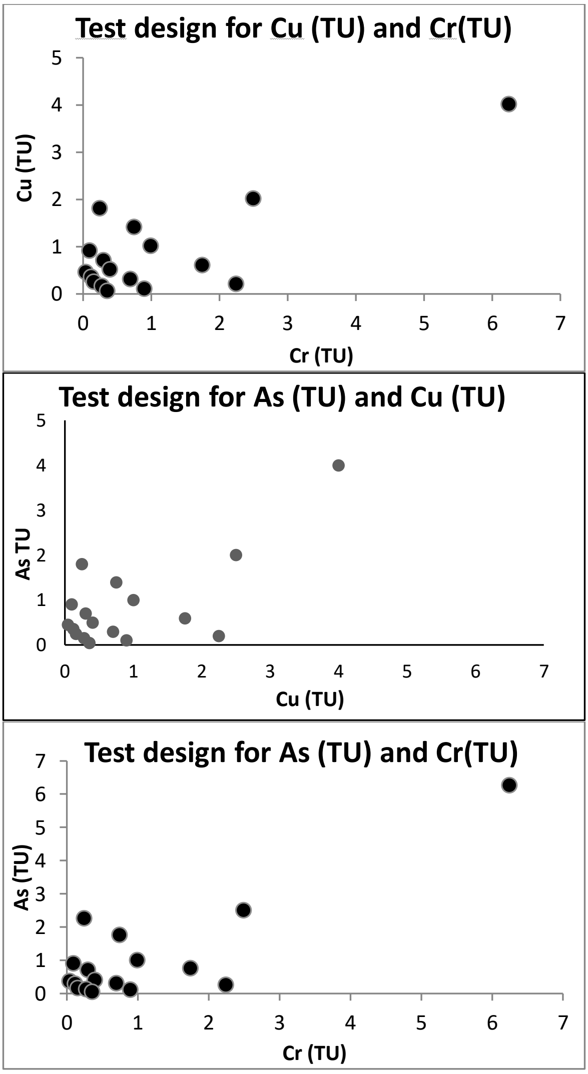

Figure 7 Nominal concentrations of the individual metals and the binary mixtures of Cu-Cr (top), Cu-As (middle) and As-Cr (bottom) based on expected toxic strengths of 0.25, 0.5, 1, 2 and 4 TU for Cu and 0.16, 0.4, 1, 2.5 and 6.25 for Cr and As. The mixture tested had toxicant ratios of 1:1, 9:1, 1:9, 1:3 and 3:1.

3.4.3 Calculating the toxicity of single metals

Single metal toxicity data were fitted to logistic and, if applicable, hormetic dose-response models. The logistic models for estimating EC_{50} and EC_{10} is given in equations 5 and 6, respectively.

$$y = \frac{y_{max}}{1+\left(\frac{c}{EC50}\right)^{slope}} \quad (5)$$

$$Y = \frac{Y_{max}}{1+\left(\frac{10}{90}\right)*\left(\frac{c}{EC10}\right)^{slope}} \quad (6)$$

The hormesis model (Van Ewijk and Hoekstra, 1993) is presented in equations 7 and 8 for EC_{50} and EC_{10} estimates, respectively.

$$y = \frac{y_{max}(1+fc)}{1+(2f*EC50+1)*\left(\frac{c}{EC50}\right)^{slope}} \quad (7)$$

$$Y = \frac{Y_{max}(1+f*c)}{1+\left(\frac{10}{90}\right)*(2fEC10+1)*\left(\frac{c}{EC10}\right)^{slope}} \quad (8)$$

In these equations, Y_{max} is the maximum response in the uninhibited control, c the exposure concentration, EC_{50} the concentration reducing the response by 50% compared to the control, f the hormesis parameter, and slope indicates the steepness of the dose-response curve. Values for these parameters and corresponding 95% confidence intervals were obtained by using the nonlinear fitting procedure in SPSS.

3.4.4 Mixture toxicity analysis

Mixture toxicity data were analyzed using the MIXTOX model developed by Jonker et al. (2005). This model allows for comparing observed data with mixture effects expected using the concentration addition (CA) and the independent action (IA) reference models. The model was applied for every binary mixture and for every metal pool (total, and water or 0.01M $CaCl_2$ extractable concentration) to assess mixture effects on the reproduction of *E. andrei*. It was first tested for possible deviations from the reference model. If deviations were seen, the CA and IA models were extended with deviation functions including extra parameters to describe synergistic/antagonistic, dose-level and dose-ratio dependency (Loureiro et al., 2010). Full details of the MIXTOX model can be found in Jonker et al. (2005). Data were fitted to the model using the solver function in Microsoft EXCEL.

3.4.5 Partitioning of metals

To assess metal partitioning in the soils, Freundlich sorption isotherms (Travis and Etnier 1981) were fitted to the measured total and extractable concentrations as shown in equation 7:

$$C_s = K_f * C_{ext}^n \tag{7}$$

Where C_s is total concentration in soil (mg/kg dry soil), C_{ext} concentration in the H_2O or $CaCl_2$ extract (mg/L), K_f the Freundlich sorption constant $((L/kg)^n)$ and n indicates the deviation from linearity. K_f and n were estimated from linear plots of log C_s versus log C_{ext}, in which control values were omitted.

3.5 Ecological risk assessment, TRIAD approach

In article I the TRIAD approach was used to assess the ecological risk of chromated copper arsenate (CCA) compound in old wood impregnation site in Hartola, Southern Finland. The TRIAD approach was applied for the 15 squares and used data from:

a) Total soil concentrations (mg/kg, dw) compared to toxicity data

Total soil concentrations measured in soils from the study site were related to data on the toxicity of CCA metals to earthworms and enchytraeids, with the lowest half maximal effective concentration (EC_{50}; reproduction) and 50% lethal concentration (LC_{50}) or lowest observed effect concentration (LOEC; survival) taken from literature.

b) Available metal concentrations in the soil solution (mg/L)

Only available copper concentrations could be compared with the highest and 5 times the highest 0.01 M $CaCl_2$-extractable concentrations in nonpolluted soils (Hobbelen et al. 2004).

c) Bioaccumulation in earthworms compared to control values (mg/kg, dw)

Metal levels in *Lumbricus rubellus* from the bioassays were compared with levels in earthworms exposed to nonpolluted soils and 5 times this level or, in the case of copper, to the critical body residue of 60 mg/kg dry body weight for lethal effects (Ma, 2005).

d) Bioaccumulation in plants *Lemna minor* and *Lactuca sativa*

The plant samples in which growth was significantly inhibited compared with other samples were used in this study.

e) Toxicity in the bioassays

Survival of earthworms and enchytraeids was compared with acceptable control mortality (10% and 20%, respectively) and serious effects (50%). For plants, samples were highlighted in which growth of *Lactuca sativa* (soil) or *Lemna minor* (H_2O extracts) was significantly inhibited compared with control or reference samples.

Results from articles II and III support the TRIAD approach applied in article I. In article II we measured bioavailability of Cu, Cr and As using uptake and elimination kinetic approach to asses potential risk to ecosystem. In article III we obtained data on the toxicity of the CCA metals, single and in binary mixtures. We evaluated the potential risk of the CCA metals in mixtures in relation to their sorption to the test soil.

4 RESULTS AND DISCUSSION

4.1 Study site

In article II, we took advantage of analyzed data from article I to establish four sampling sites, each measuring 1.5 m x 1.5 m: control (C), low (L), medium (M) and high (H) (Kilpi-Koski et al., 2019). The control square was moved to next to our site because the total concentrations of Cr, Cu and As were the same range as in squares 10 – 12 and higher than in squares 1 – 3 reported by Karjalainen et al. (2009). The low site in article II in fact was the reference site of article I. The medium site was found near the drying area and the high site was the same area were squares 4 – 6 of article I were located. We made these changes to create a more consistent concentration gradient and to avoid large scatter in soil concentrations.

4.2 Soil properties

The contaminated site was located in a very typical Finnish forest, consisting of a Norway spruce (*Picea abies* L.) stand of the Oxalis-Myrtillus site type (Cajander, 1949), in Hartola southern Finland. In Table 1 the soil properties measured for articles I and II are shown.

Table 1. *Soil properties of our study site in Hartola, southern Finland. Shown are parameters reported in articles I and II. The values from article I are ranges measured in the gradient of CCA pollution; the values from article II are mean values with standard deviation, OM = organic matter, WHC = water holding capacity.*

Article I	pH_{CaCl_2}		%OM	WHC	Moisture content (%)			
	3.58 – 5.03		16.4 – 56.5	119 – 62	29.9 – 9.1			
Article II Site	pH_{CaCl_2} (n = 3)	pH_{H_2O} (n = 3)	%OM (n = 3)	WHC (n = 10)	Moisture content (%) (n = 5)	% clay (< 8 µm) (n = 5)	% silt (8-64 µm) (n = 5)	% sand (64-2000 µm) (n = 5)
Control	3.39±0.05	4.30±0.10	30.8±2.63	325±77	49.6±2.94	5.1±1.6	11.1±1.7	83.7±3.3
Low	3.89±0.01	4.95±0.10	23.9±6.62	271±124	50.5±9.62	4.1±0.8	10.3±0.8	85.6±1.6
Medium	4.32±0.07	5.53±0.02	31.8±4.23	277±56	93.8±11.6	4.8±0.6	12.6±1.6	82.6±1.8
High	4.53±0.08	5.68±0.07	20.8±5.50	238±105	41.6±11.2	3.1±2.7	6.5±1.7	90.4±4.2

In article II, the impact of the soil properties on the bioavailability of Cr, Cu and As and their partitioning in the soil compartment was discussed in more detail. The field soils near Hartola were acidic with pH-$CaCl_2$ between 3.39 and 5.03. Under acidic conditions, the mobile Cr(VI) is reduced to the stable and less toxic Cr(III) (Kumpiene et al., 2008; Sivakumar and Subbhuraam, 2005). In soil, copper is bound to organic matter. When pH increases the sorption of the free Cu^{2+} ion on solid organic matter increases (Degryse et al., 2009) and Cu also becomes more strongly bound to oxide surfaces (Khaodhiar et al., 2000). Peijnenburg et al. (1999) did not observe significant uptake of arsenic in earthworms at $pH_{CaCl_2} < 6$ and at $pH_{CaCl_2} > 6.75$. At low pH and high redox potential, As is mainly in the As(V) form, but when pH increases and redox potential decreases As(III) is the dominant form in soils (Masscheleyn et al., 1991) and in earthworms (Lee and Kim, 2013). Soil acidity was found to be the most important characteristic modulating the availability of As and its sorption (Balasoiu et al., 2001; Peijnenburg et al., 1999).

Many studies have shown that metals bind to organic matter and, as a consequence, are not available for uptake. Cr, Cu and As all have high affinity for binding to soil organic matter (Meharg et al., 1998; Peijnenburg et al., 1999; Marinussen et al., 1997; Speir et al., 1995). In this study, organic matter contents (OM) were high, ranging between 16 – 57 % (article I) and 21 – 32 % (article II), while the Hartola soils were sandy with low clay content. Balasoiu et al. (2001) concluded that Cu was bound to organic matter because of suitable reactive groups and retained by complexation rather than ion exchange. Chromium partitioning to organic matter was similar to that of copper (Balasoiu et al., 2001). In high organic soils, Cr and Cu are present in less mobile and less available forms (Balasoiu et al., 2001; Gupta et al., 1996; Maiz et al., 2000). Also As has high affinity for binding to soil organic matter *(*Meharg et al., 1998).

4.3 Total metal concentrations of the field soils and well water

Based on the analysis done in article I, we knew that there were high total concentrations of Cr, Cu and As, especially in the soil near the impregnation cylinders. As seen in Table 2, we did not succeed so well in establishing very clear and consistent concentration gradients, neither in article I nor in article II. In article I, the concentration gradient was quite heterogeneous, while in article II the total concentrations of Cr, Cu and As in the H and M sites were quite similar and those in both the L and C sites very low.

Table 2. *Mean (± SD) total concentrations [mg/kg dry soil] of chromium (Cr), copper (Cu) and arsenic (As) in the CCA contaminated soils from Hartola, Finland, used for the bioassays (article I) and the toxicokinetics experiment with Eisenia andrei (article II).*

Article I Study site	Total [mg/kg] (n=3/square)			Article II Study site	Total [mg/kg] (n=37-39)		
	Cr	Cu	As		Cr	Cu	As
Block 1: Squares 1 – 3	47.0±21.2	13.1±3.91	31.1±19.0	Control (C)	5.34±1.68	4.71±1.06	6.12±1.22
Block 2: Squares 4 – 6	684±916	284±439	1613±2380	High (H)	1480±355	642±180	2810±921
Block 3: Squares 7 – 9	369±237	253±205	808±688	Medium (M)	1590±247	791±140	850±225
Block 4: Squares 10 – 12	43.6±4.56	31.3±7.56	77.1±10.4				
Block 5: Squares 12 – 15	30.3±19.9	13.4±10.9	50.2±38.6	Low (L)	12.5±10.6	5.14±5.3	10.1±5.5

The Finnish upper guideline values for As, Cr and Cu are 100, 300 and 200 mg/kg, respectively (Ministry of the Environment, Finland, 2007) were exceeded in blocks 2 and 3 (article I) and the H and M sites (article II).
In article I, two well waters were used for the bioassays as well. Average total metal concentrations in the water were 0.024, 0.001 and 0.007 mg/L for As, Cr and Cu, respectively.

4.4 H_2O and $CaCl_2$ extractable concentrations

All three CCA metals were available in the soils from the study site, but this did not lead to a high bioavailability for plants (article I) or invertebrates (articles I and II). Availability of Cu, Cr and As was determined by H_2O- and 0.01 M $CaCl_2$ extraction of the field soils. Our findings of low H_2O- and 0.01 M $CaCl_2$-extractable concentrations of Cr, Cu and As in the acidic and high organic CCA-contaminated field soils from Hartola (articles I and II) can partially be explained from soil properties as mentioned earlier.

In article I, 0.01 M $CaCl_2$-extractable concentrations did not differ between the top 2 cm and the 2 – 15 cm two soil layers, but H_2O-extractable concentration was higher in the deeper layer than in the top soil. The good correlation with total metal concentrations and the variability of soil properties may be due to ageing effects. Binding of metals to soil generally tends to become stronger with time, reaching equilibrium between the soil solid phase and soil solution (pore water) in the long run (Sauvé et al., 2000). The highest H_2O and 0.01 M $CaCl_2$-extractable concentrations were found in square 4. In general, the extractable metal concentrations were lower in the top soil than in the deeper layer because new, clean litter has accumulated in the top soil after the cessation of the wood-impregnation activities. The H_2O-extractable metal concentrations gave a good prediction of body concentrations of the CCA metals in soil invertebrates and the mortality of the earthworm *Lumbricus rubellus* exposed for 4 weeks in Hartola field soil.

In article II, H_2O and 0.01 M $CaCl_2$ extractable metal concentrations were below the detection limit for the control and low contaminated soils. For Cr and As, water-extractable concentrations in the high and medium contaminated soils were slightly higher than the $CaCl_2$-extractable concentrations. For copper, the difference between water- and $CaCl_2$-extractable concentrations in these soils was small and not consistent. In our test soils, the available concentrations of Cr, Cu and As were very low in the low contaminated soil and in general less than 1% of the metal was available in the medium and high contaminated soils, except for the water extractability of As which was 1 – 2%. The slightly higher metal availability in the high contaminated soil compared to the medium contaminated soil can be attributed to the higher organic matter and clay content of the latter. The fact that for Cr and As water-extractable concentrations were higher than $CaCl_2$-extractable concentrations suggests these elements were not present as cations in the CCA-contaminated field soils (article II) as indicated also in article I.

In article III, we assessed the toxicity of Cu, Cr and As, single and in binary mixtures, using OECD artificial soil in which bioavailability of metals may be different than in the field soils (Peijnenburg et al., 1999). Metals are more bioavailable in freshly spiked artificial soils than in field-contaminated soils (Spurgeon and Hopkin, 1995). Soil properties have a huge impact on the solubility of metals as mentioned earlier. H_2O and 0.01 M $CaCl_2$-extractable concentrations of Cu, Cr and As in the OECD artificial test soil were similar and increased with increasing total soil concentrations. This was unexpected as usually for cationic metals the 0.01 M $CaCl_2$-extractable concentrations are higher than H_2O-extractable concentrations because of cation exchange effects (Giska et al., 2014; Hobbelen et al., 2006). We do not have a clear explanation why extractable concentrations were quite similar in our test soils. The most probable reason is that a freshly spiked artificial soil has a lower sorption capacity and that the ageing process was not completed when we analysed our soils. Kim et al. (2015), Langdon et al. (2002), Mesuere and Fish (1992), Schultz et al. (2004) and Balasoiu et al. (2001) found that Cu, Cr and As are forming complex ions influenced by soil properties. However, despite of possible complex formationing capabilities, As showed rather high availability in both H_2O and 0.01 M $CaCl_2$ extracts. This can be explained by the fact that As occurs as an anionic species and thus the yield is not affected by the extraction method.

In article III, we calculated Freundlich K_f parameters to describe the sorption of Cu, Cr and As added to the artificial soil, single and in binary mixtures. The Freundlich sorption isotherms for Cu had n values <1 suggesting a concentration effect, especially with As and Cr at a 30:70 Cu:metal ratios. At the lower concentrations Cu sorption was low at the 10:90 Cu:metal ratio, but increased for all other mixtures with As and Cr. The sorption K_f values for Cr with Cu and As were quite similar, lower sorption at 10:90 Cr/Me ratios and increased at the higher Cr concentrations. The sorption of As in different Cr/Me ratios was quite similar, but n values indicated possible complexation of the metals (n>1). Cr and As can form less soluble complexes (Kües, 2007). Sorption of As increased with decreasing Cr level so increasing ratio of As/Cr. This is supported by Buchter et al. (1989) who observed greater retention for As compared to Cr.

4.5 Bioassays

A set of bioassays was applied to assess the contaminated field soil (articles I and II), its aqueous extracts and well water (article I) from the Hartola site. Biological tests were also applied to assess the bioavailability of the CCA metals in the field soils (article II) and their toxicity, single and in mixtures, in the OECD artificial soil (article III). Exposures used the plants *Lactuca sativa* and *Lemna minor*, the earthworms *Lumbricus rubellus* (article I) and *Eisenia andrei* (articles II and III) and the enchytraeid *Enchytraeus albidus* (article I).

4.5.1 Single metal toxicity

Phytotoxicity tests

Plant growth was affected by CCA contamination interfering with seed germination and root elongation of *L. sativa* and *L. minor*, both in well water and in contaminated field soils. Although the As concentration in well water was 2-fold above the maximum acceptable limit of 0.01 mg/L (Ministry of Social Affairs and Health, Finland, 2000), it did not significantly correlate with growth or seed germination of *L. sativa* ($p = 0.622$), but it did explain for the effects on *L. minor* which was more sensitive. Its root elongation and leaf numbers increased linearly with increasing dilution of the well water. Tests with *L. sativa* on contaminated soil did not go well, mainly because quartz sand controls did not contain organic matter. Root elongation was more sensitive than seed germination and correlated significantly with total ($p = 0.0259$) and H_2O-extractable ($p = 0.0502$) As concentrations in the soils. *L. minor* growth differed significantly from the control in all diluted soil extracts (Mann-Whitney, $p < 0.05$).

Invertebrate survival and reproduction

In article I, neither the total metal concentration nor soil properties (pH-$CaCl_2$ 3.58 – 5.03 and organic matter content 16.4 – 56.5 %) did have significant effects on earthworm survival (2 weeks, $p = 0.825$ and 4 weeks, $p = 0.458$). According to the multiple regression analysis the earthworm mortality is attributed to the combination of metals present ($p = 0.0295$). The mortality of *E. albidus* was low and did not correlate with soil properties ($p = 0.378$), but survival of *E. albidus* did correlate with body metal concentrations. Metal bioaccumulation in *L. rubellus* was measured only after 4 weeks exposure. Cr accumulated the most ($p = 0.024$). As indicated by Neuhauser et al. (1995), exposure time should be longer than 28 days to reach equilibrium and allow for obtaining more accurate results. The number of juveniles of *E. albidus* varied a lot from 0 – 45 per test container and correlated with OM content of the test soils ($p = 0.0311$).

In article II, survival of the earthworm *E. andrei* was high during the 42-day experimental period, with only 3.61%, 2.41%, 8.43% and 13.3% mortality in control, low, medium and high soils, respectively.

In article III, control survival of *E. andrei* was 100%, while the number of juveniles produced in the controls was 31.6 ± 4.04 (±SD, $n = 5$), so meeting the validity criteria set by the OECD guideline 222 (OECD, 2004). Cu and Cr did not affect earthworm survival at the concentrations tested, but As caused a dose-related increase of mortality with an estimated LC_{50} of 92.5 mg As/kg in the OECD artificial soil. Cu and Cr showed a hormetic effect on earthworm reproduction at low concentrations, and a dose-related decrease at higher concentrations. In the literature we found hormetic effects of Cu on the growth and development of juveniles of *L. rubellus* (Spurgeon et al., 2004). No indications for hormetic effects of Cr on earthworms were found in the literature. Arsenic did not show hormetic effect, but it did affect the reproduction of E. fetida at intermediate total concentrations (<45 mg/kg, dw) in low organic mining area soils (Neaman et al., 2012; Bustos et al., 2015).

Effect concentrations (EC_{50} and EC_{10})

Based on results in article III we calculated effect concentrations (EC_{50} and EC_{10}) which were compared with values from the literature. The EC_{50} for the effects of Cu on the growth of *E. andrei* in OECD artificial soil was > 100 mg/ (dw) (van Gestel et al., 1991). In a Portuguese natural soil, the EC_{50} for effects on the reproduction of *E. andrei* was 130.9 mg/kg (dw) and the EC_{20} was 73 mg/kg (dw) (Caetano et al., 2016). These values support our EC_{50} and EC_{10} of 154 and 74 mg Cu/kg (dw), respectively. We reported also EC_{50} values based on H_2O and $CaCl_2$-extractable concentrations 9.7 and 9.0 mg/kg, respectively and EC_{10}s of 3.60 and 3.92 mg/kg, respectively. In the literature, we found only a $CaCl_2$-extractable EC_{50} of 1.84 mg/kg for Cu effects on the reproduction of *E. crypticus* in OECD artificial soil (Posthuma et al., 1997), which is somewhat lower than our values.

Our EC_{50} and EC_{10} for the effects of Cr of 449 and 343 mg/kg are in the same range as the 21-day EC_{50} of 892 mg Cr/kg for effects on the cocoon production of *E. fetida* and the 42-day EC_{50} of 637 mg Cr/kg for effects on the reproduction of *E. albidus* (Lock and Janssen, 2002b).

The EC_{50} and EC_{10} values for As of our study were 9.08 and 5.80 mg/kg, dry soil, respectively, which is lower than the values reported by Romero-Freire et al. (2015) but in the same range as the values found by Lock and Janssen (2002c) and Bustos et al. (2015). Characteristics of our artificial soil (pH and OM) were different than those of the soils used by Romero-Freire et al. (2015) which may have caused the differences in test results.

Bioavailability of Cu, Cr and As

In article I, As was found to cause inhibition of plant growth while it also accumulated in the invertebrates. Also chromium had effects on *E. albidus*. In article II, we wanted go a little further by investigating the bioavailability of all three metals to *E. andrei* applying a toxicokinetic approach. In the low (L) contaminated soils, no uptake of Cr, Cu and As was seen. In the medium (M) and high (H) contaminated soils, uptake and elimination kinetics of Cr and Cu was very fast as shown by the high uptake (k_1) and elimination (k_2) rate constants. Equilibrium was reached within 1 day. Similar patterns for Cu and Cr were found by Peijnenburg et al. (1999).

Question is why the uptake and elimination patterns of Cu and Cr are so similar. Copper is an essential metal, playing an important role in some metabolic processes, like the release of insulin from tissues when needed for the usage of sugars, proteins and fats (Shrivastava et al., 2002). Cr also is considered an essential metal. When looking for chemical characteristics of Cu and Cr, they are very similar. They both are cationic in acidic environments, their size is similar and they are Lewis acids which bind strongly to organic matter. This may explain why these two metals have a similar uptake and elimination pattern *in E. andrei* like other essential metals. However, the metabolic routes of Cu and Cr are different in earthworms. Spurgeon and Hopkin (1999) support our results of the fast elimination of Cu, which may indicate that copper is detoxified mainly by excretion. Earthworms can regulate their copper body concentration (Fisker et al., 2011) using a metallothionein protein, although Cu itself it is not so active in inducing metallothionein-gene expression. Chromium(VI) is accumulated inside cells through the same membrane channels used for the transfer of isoelectric and isostructural anions, like SO_4^{2-} and HPO_4^{2-}. Glutathione, which is present in high concentrations, plays an important role in the intracellular metabolism of Cr(VI) (Connett and Wetterbahn, 1986 ; Codd et al., 2001).

Uptake and elimination patterns of As were different than those for Cu and Cr. Arsenic showed very slow kinetics in *E. andrei* exposed to M and H contaminated soils. Steady state was not reached within 21 days of exposure, and very low k_1 and k_2 values were found. This is supported by the findings of Lee and Kim (2013) and Peijnenburg et al. (1999). Totally opposite findings compared to our study were found by González-Alcaraz and van Gestel (2016). They reported that As body concentrations of *E. andrei* increased very fast, with steady-state being reached in 1 – 3 days and the elimination rate constant was much higher. This may be caused by different soil properties with high pH-$CaCl_2$ and low organic matter contents in their soils. Metal intake in earthworms is mainly via the alimentary route (Morgan et al., 1994; Langdon et al., 1999). Arsenic is sequestered as As-thiol complexes (Morgan et al., 1994) but also other metal binding proteins may be involved as well, like metallothioneins (Langdon et al., 2005) causing the bioaccumulation of As in the earthworms (Lee and Kim, 2013). Earthworms may have restricted capabilities to eliminate As (Fisher and Koszorus 1992) explaining the high As accumulation in *E. andrei* in our study.

Estimated from uptake and elimination rate constants, bioaccumulation factors (BAFs) for the accumulation of Cr and Cu were below 0.1. Similar BAF values for Cu accumulation in *L. rubellus* exposed to two different soils in UK (0.060 and 0.049) were found by Langdon et al. (2001b) and for Cr (0.031 – 0.047) in *E. andrei* exposed to freshly spiked artificial soil by van Gestel et al. (1993). In our study, the BAF values for As of 1.8 and 0.54 for M and H sites, respectively, show that As is accumulated by *E. andrei*. Langdon et al. (2001a) suggested that different earthworm species can detoxify As in a different way explaining for their very low BAF values. Peijnenburg et al. (1999) reported a BAF of 0.64 for As uptake in *E. andrei* exposed to Dutch field soil based on steady state concentrations. This value is similar to our data indicating that As speciation in their soils was similar to our soils.

In article I, BAF was calculated from the ratio of body and soil metal concentrations. BAFs of Cr, Cu and As for the earthworm *L. rubellus* were less than 1 suggesting that no bioaccumulation occurred. For *E. albidus,* BAFs were > 1 indicating that bioaccumulation occured. For aquatic organisms or for exposure from an aqueous medium, bioconcentration factors (BCFs) are estimated as the ratio of body concentration and solution-phase metal concentrations. Calculation of BCFs for oligochaetes may better explain the accumulation of Cu, Cr and As because they are primarily exposed through dermal contact with the soil solution (pore water) (Vijver et al., 2003). BCFs calculated using water-extractable concentrations were high for both the earthworms (40.9 – 208) and the enchytraeids (58.5 – 1190).

4.5.2 Binary mixture toxicity

Soil properties and metal characteristics influence the reactions between metals in contaminated soils. In article I, *L. minor* was more sensitive for the metal mixtures than *L. sativa*, which may be caused by differences in metal uptake. This was the first indication that metal mixtures may influence the risk of CCA-contaminated soils. In article III, we wanted to study how mixture toxicity contributes to or explains for the risk of CCA metal contaminated soils. We exposed *E. andrei* to three binary mixtures: Cu – As, Cu – Cr and Cr – As, at various concentration ratios. Mixture effects were compared to the effects of the single metals, and related to total and H_2O and 0.01 M $CaCl_2$ extractable metal concentrations in the OECD artificial test soil.

We could not analyze the mixture effects on the earthworm survival using the MIXTOX model by Jonker et al. (2005) because only in the mixtures with As significant and dose-related mortality occurred. We analyzed the effects of the binary metal mixtures on the reproduction of *E. andrei* using the reference models of concentration addition (CA) and independent action (IA) included in the MIXTOX model. Table 3 summarizes the results on the toxicity of the binary mixtures of the CCA metals to the reproduction of *E. andrei* in OECD artificial soil.

Table 3. *Summary of the results on the toxicity of binary mixtures of As, Cr and Cu to the reproduction of Eisenia andrei in OECD artificial soil, based on total and extractable concentrations (H_2O and 0.01 M $CaCl_2$) in the test soil.*

mixture	expression of exposure	CA	IA
Cu-As	Total	antagonism	additivity
	H_2O extract	antagonism	additivity
	$CaCl_2$ extract	additivity	synergism for S/A, no further DR and DL deviations
Cu-Cr	Total	antagonism	additivity
	H_2O extract	antagonism; DR shows that the mixture is more antagonistic at increasing Cu concentration	additive, but tendency to DR with antagonism at increasing Cu concentration
	$CaCl_2$ extract	additivity	synergism for S/A, no further DR and DL deviations
Cr-As	Total	antagonism; DR, antagonistic at increasing Cr concentration	antagonism; DR, antagonistic increasing Cr concentration
	H_2O extract	antagonism	antagonism
	$CaCl_2$ extract	antagonism	antagonism

As seen in the Table 3, antagonistic effects were found for all binary mixtures of Cu-As, Cu-Cr and Cr-As when tested against the CA model and related to measured total soil concentrations. Spurgeon and Hopkin (1995) found antagonistic effects for mixtures of Cu, Zn, Cd and Pb when exposing *E. fetida*. The effects on the cocoon production of the earthworm *A. caliginosa* exposed to mixtures of Cu, Cd and Zn were antagonistic against the CA model (Khalil et al., 1996). The toxicity of Zn-As was overall significantly antagonistic for barley when tested against the CA model and less strongly antagonistic against the IA model (Guzman-Rangel et al., 2018) and for rice effects were antagonistic when exposed to a mixture of As and Cu. Antagonistic effects of the studied binary mixtures were also found when expressing toxicity on the basis of H_2O and 0.01 M $CaCl_2$-extractable concentrations, and related against the CA model. Dose-ratio dependent deviation from additivity was detected with increasing Cu levels causing stronger antagonism in the Cu-Cr mixture and increasing Cr levels causing stronger antagonism in the Cr-As mixtures when related to total concentrations at low Cr concentrations for both reference models of CA and IA. No dose level-dependent deviations were found in our tests.

4.5.3 TRIAD approach assessing the risk in Hartola soil

In this thesis, our main aim was to investigate the possible risk of CCA-contaminated field soils in Hartola, Finland using a combination of bioassays, a toxicokinetic approach to assess the bioavailability of chromium, copper and arsenic and a binary mixture toxicity approach. The TRIAD approach was chosen to assess the possible risks for the ecosystem.

The 1st line of evidence was to compare total metal concentrations in the contaminated field soils to toxicity data and given target or trigger values for the three CCA metals. The 2nd line of evidence consisted of bioassays in which organisms were exposed to the contaminated field soil. The 3rd line of evidence contained information on ecosystem health through the determination of the abundance of enchytraeids and nematodes and the bioaccumulation of metals in horsetail (*Equisetum* spp.) growing abundantly at the contaminated area. Table 4 shows the 1st and 2nd lines of evidence according to results of article I, Tables 5 and 6 show results from article II and article III, respectively supporting the 1st and 2nd lines of evidence of the TRIAD approach performed in article I (Table 4).

Table 4. *The 1st and 2nd lines of evidence of the TRIAD approach applied to CCA contaminated soil in Hartola, Finland, based on results reported in article I.*

Article I																		
Parameter	Metal or endpoint	Criteria		Sample/squares														
		low	high	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Total soil concentration (mg/kg, dw) compared to toxicity data	As	23	96	g	y	y	x	x	x	x	x	g	y	y	y	y	g	y
	Cr	155	1000	g	g	g	x	g	y	y	y	g	g	g	g	g	g	g
	Cu	250	1000	g	g	g	y	g	g	y	y	g	g	g	g	g	g	g
Available soil conc (mg/L)	Cu-$CaCl_2$	0.2	2	y	y	y	x	y	y	x	y	y	x	y	y	y	g	g
Bioaccumulation in earthworms compared to control values (µg/g, dw)	As	18	90	g	y	y	x	y	y	x	y	y	y	y	y	y	g	y
	Cr	25	125	g	g	g	x	g	g	x	y	g	g	g	g	g	y	x
	Cu	15	60	g	g	g	x	y	g	x	g	g	y	y	g	g	g	y
Toxicity in bioassays	Earth worm	<90 %	<50 %	y	y	y	x	y	y	y	y	g	y	y	y	g	g	y
	Enchy traeid	<80 %	<50 %	g	y	y	y	g	g	g	g	g	y	g	g	g	y	g
	Lactuca sativa		sign.	g	x	x	x	g	g	g	g	g	g	g	x	g	g	g
	Lemna minor		sign.	g	g	g	x	g	g	g	g	g	g	g	g	g	g	g
Conclusion: The 1st line of evidence of the TRIAD approach used toxicity data for earthworms and enchytraeids and compared total soil concentrations with EC_{50}, LC_{50} and LOEC values from the literature. These findings suggest that As is the primary source of toxicity in several sample squares. To expand our review to the 2nd line of evidence comparing bioaccumulation levels with background levels in animals from nonpolluted soils, it showed that As is causing the highest risk. Overall review of the bioassay results confirms that the highest ecological risk is found at the most polluted square 4 which matches with the high (H) site sampled for article II.																		

*Green indicates that concentrations or effects do not exceed trigger values. Yellow and red indicate exceedance of the lower and higher criteria, respectively.

Table 5. *Uptake and elimination kinetics of CCA metals in the earthworm Eisenia andrei exposed to Hartola soils, reported in article II and compared with criteria from article I.*

Article II							
		Criteria			In Hartola soils		
		Low	High	Control	High	Medium	Low
Total soil concentration (mg/kg, dw) compared to toxicity data	As	23	96	6.12	2810	850	10.1
	Cr	155	1000	5.34	1480	1590	12.5
	Cu	250	1000	4.71	642	791	5.14
Bioaccumulation in earthworms (BAFs)	As			<	0.54	1.8	<
	Cr			<	0.029	0.036	<
	Cu			<	0.067	0.086	<
Uptake rate constant k_1 (kg soil/kg worm/day)	As				0.0065	0.011	-
	Cr				0.71	0.27	-
	Cu				0.16	0.19	-
Elimination rate constant k_2 (day^{-1})	As				0.012	0.0062	-
	Cr				24.6	7.6	-
	Cu				2.4	2.2	-

Conclusion:
Compared to the literature values used in Article I, total soil concentrations were higher indicating potential risk for the ecosystem in the H and M areas. Analysis of H_2O and 0.01 M $CaCl_2$ extractable concentrations showed that metals were available for *E. andrei*. In the H and M areas bioaccumulation of As was seen supported by BAF values of 0.54 and 1.8, respectively. In article I, bioaccumulation of metals was seen in *L. rubellus*. Uptake rate constants of Cr and Cu were > 0.1 kg soil/kg worm/day and elimination rate constants >10 day^{-1} showing fast uptake and very fast elimination. After equilibrium was settled the background levels were reached within 1 day. Uptake and elimination rate constants of As indicated very slow uptake and elimination and the steady-state was not reached during the test period (21 d).

Table 6. *Results on the toxicity of CCA metals, single and in binary mixtures, to the earthworm Eisenia andrei in OECD artificial soil, taken from article III and compared with values from the literature.*

<table>
<tr><td colspan="5">Article III</td></tr>
<tr><td></td><td></td><td colspan="3"></td></tr>
<tr><td rowspan="2">Single metal toxicity</td><td rowspan="2"></td><td colspan="2">Values from the literature</td><td rowspan="2">results of our study</td></tr>
<tr><td>Article I</td><td>Article III</td></tr>
<tr><td rowspan="3">EC_{50} total conc., logistic model</td><td>As</td><td>96</td><td>10.8***</td><td>9.08</td></tr>
<tr><td>Cr</td><td>1000</td><td>892**</td><td>449</td></tr>
<tr><td>Cu</td><td>1000</td><td>>100*</td><td>154</td></tr>
<tr><td colspan="5">Conclusion:
The toxicity of As was unexpectedly high compared to values found in the literature, indicating potential risk for the soil ecosystem. EC_{50}s for Cr and Cu were in agreement with literature values.</td></tr>
<tr><td></td><td></td><td colspan="3">Conclusion:</td></tr>
<tr><td rowspan="3">Binary mixture toxicity based on total soil concentrations (mg/kg, dw)</td><td>Cu-As</td><td colspan="3">Overall antagonism when tested against the concentration addition (CA) model, additive when data were fitted to the independent action (IA) model.</td></tr>
<tr><td>Cu-Cr</td><td colspan="3">Overall antagonism when tested against the CA model, with significant dose-ratio (DR) dependent deviation. Synergism is shown when Cr is the dominating element.</td></tr>
<tr><td>Cr-As</td><td colspan="3">Both the CA and IA models indicated antagonism. Synergism was seen when As was dominating the mixture.</td></tr>
<tr><td></td><td></td><td colspan="3">Conclusion:</td></tr>
<tr><td rowspan="3">Binary mixture toxicity based on H_2O-extractable concentrations (mg/kg, dw)</td><td>Cu-As</td><td colspan="3">Overall antagonism when tested against the CA model, additive when data were fitted to the IA model.</td></tr>
<tr><td>Cu-Cr</td><td colspan="3">Antagonism when tested against the CA model and additive when related to the IA model. Dose-ratio dependency with antagonism at increasing Cu levels in the mixture.</td></tr>
<tr><td>Cr-As</td><td colspan="3">Antagonistic against the CA and IA models.</td></tr>
<tr><td></td><td></td><td colspan="3">Conclusion:</td></tr>
<tr><td rowspan="3">Binary mixture toxicity based on 0.01 M $CaCl_2$-extractable concentration (mg/kg, dw)</td><td>Cu-As</td><td colspan="3">Additive when tested against the CA model and synergistic when related to the IA model.</td></tr>
<tr><td>Cu-Cr</td><td colspan="3">Additive when tested against the CA model and synergistic according to the IA model.</td></tr>
<tr><td>Cr-As</td><td colspan="3">Antagonistic when tested against the CA and IA models.</td></tr>
</table>

* EC_{50} the effect of Cu on the growth of *E. andrei* (van Gestel et al., 1991)

** EC_{50} for the effects of Cr on the cocoon production of *Eisenia fetida* (Lock and Janssen, 2002b)

*** EC_{50} for the effects of As on the cocoon production of *E. fetida* in OECD artificial soil (Lock and Janssen, 2002c)

For the 3rd line of evidence, we measured the numbers of enchytraeid and nematodes in the contaminated soil and compared it to the average of 45 enchytraeid and 500 nematodes per gram of dry soil in control plots (Setälä, H., personal communication 2006). The numbers of enchytraeids and nematodes in the Hartola soil samples were much lower at 0.30 – 6.18/g soil and 4.37 – 40.2/g soil, respectively. The number of nematodes correlated significantly with soil organic matter content ($p = 0.0008$), while the number of enchytraeids did not correlate with metal concentrations or soil properties ($p > 0.05$). The abundance of enchytraeids and nematodes was lower in the metal-contaminated Hartola soils than in unpolluted forests from southern Finland (Haimi and Mätäsniemi, 2002). Also Yeates et al. (1994) found that populations of enchytraeids and nematodes were greatest at lower concentrations of Cu, Cr and As. These finding suggest the nematode and enchytraeid numbers at the Hartola site were lower than expected, and that CCA contamination may be one of the reasons but not the only explanation for this.

The total As concentrations in shoots (14.5 – 27.0 mg/kg, dw) and roots (5.55 – 96.2 mg/kg, dw) of horsetail (*Equisetum* spp.) could not be explained by the total As concentrations in the soil from the plant squares (46.1 – 1850 mg/kg, dw; $p = 0.735$). The extremely large number of horsetails growing at the Hartola site indicates their potential tolerance to arsenic.

5 CONCLUSIONS

This research aimed at evaluating the eco(toxico)logical risk of chromium, copper and arsenic for the Finnish forest ecosystem at Hartola, southern Finland by using a combination of bioassays, uptake and elimination kinetics and binary mixture toxicity studies. Based on the results published in three articles, it can be concluded that at the Hartola study site metals are accumulated in organisms leading to an increased eco(toxico)logical risk. All three metals are bioavailable in the Hartola soils, but As is more available and therefore making a greater contribution to the toxicity of Hartola soils than Cu and Cr. The higher bioavailability of As was evidenced by its higher bioaccumulation in the test organisms.

Total metal concentrations in the Hartola soils often exceeded the LC_{50} or EC_{50} values reported in the literature and were also higher than the EC_{50} and EC_{10} values for Cu, Cr and As reported in article III, indicating hazards to the Hartola soil ecosystem (article I). Arsenic was the most toxic metal in this study site. This metalloid may steadily accumulate in earthworms, at a slow rate but reaching high concentration levels and causing risk for the Hartola soil ecosystem (article II).

The mixture toxicity data was analysed using the MIXTOX model by relating effects against the reference models of concentration addition (CA) and independent action (IA). H2O and 0.01 M CaCl2-extractable concentrations of Cr and Cu were too low to cause growth effects on *Eisenia andrei*. Arsenic had a high availability in the OECD artificial soil as shown by the high H2O and 0.01 M CaCl2-extractable concentrations, explaining for its unexpected high toxicity for *E. andrei*. The binary toxicity mixture studies (article III) showed that the CCA metals interact with each other, generally chemical interactions led to a lower availability of the metals in the mixtures.

When combining all results from articles I and II, it may be concluded that the adverse effects on the Finnish forest ecosystem due to CCA metal contamination are mainly due to arsenic. The binary mixture toxicity experiment (article III) supports this conclusion article.

This research clearly demonstrated the bioavailability of Cu, Cr and As, but it was unable to answer questions regarding the interactions between the three metals in mixtures. To understand better the implications of this question, future studies should assess mixtures containing all three metals and taking into account (differences in) the speciation of these metals in the field soils and the artificial test soil used in article III of this study.

Based on the conclusions described in articles I, II and III, we covered the three lines of evidence required by the TRIAD approach: chemistry, toxicology and ecology, although the ecological approach is less investigated in this thesis. Our study shows that together with TRIAD and Decision Support System (DSS) approaches, the bioaccumulation and mixture toxicity approaches may offer a more systematic way of assessing the ecotoxicological risk of metal-contaminated soils. The TRIAD approach can be used to assess the eco(toxico)logical risk of chromated copper arsenate contaminated soils.

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